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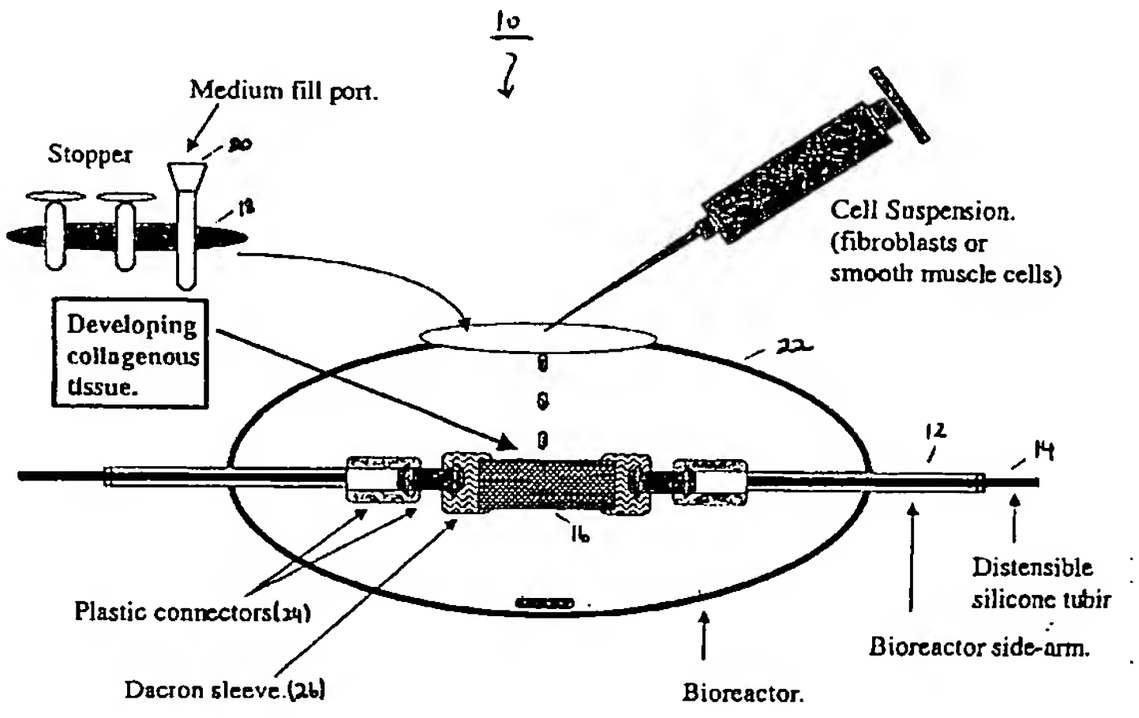

(57) **Abstract:** New methods for producing tissue engineered constructs and engineered native tissues are disclosed. The methods include producing a tissue engineered construct bz growing cells *in vitro* on a substrate and then decellularizing the construct to produce a decellularized construct consisting largely of extracellular matrix components. The construct can bu used immediatley or stored unitl needed. The decellularized construct can be used for further tissue eingineering, which may include seeding the construct with cells obtained from the intended receipient of hte construct. During any of the growth phases required for production of the construct, the developing construct maz be subjected to various tissue engineering steps such as applciation of mechanical stimuli including pulsatile forces. The methods also include producing an engineered native tissue by harvesting tissue from an animal or human, performing one or more tissue engineering steps on the tissue, and subjecting the tissue to decellularization. The decellularized, engineered native tissue may then be subjected to further tissue engineering steps.

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DECELLULARIZED TISSUE ENGINEERED CONSTRUCTS AND TISSUES

GOVERNMENT SUPPORT

The U.S. government has a paid-up license in this invention and the right under certain circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of grant number HL-03492 awarded by the NHLBI.

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application 60/225,698, filed August 16, 2000, the contents of which are hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

Tissue damage, dysfunction, or loss is a feature of a wide variety of medical conditions. Atherosclerosis, in which formation of fatty plaques in blood vessel walls leads to narrowing of the vessels, is one well-known example. Accidents frequently result in damage to tendons, ligaments, and joints. Degenerative diseases such as arthritis represent another source of injury to such tissues. Systemic diseases such as diabetes, cancer, and cirrhosis are yet another cause of organ destruction or dysfunction.

In many of the situations described above, replacement of the damaged tissue or organ is the best or even the only option. Transplantation from human donors (either live or cadaveric) has enjoyed significant success, and procedures such as liver, heart, and kidney transplants are becomingly increasingly common. However, the severe shortage of donors, the complexity of harvesting organs and delivering them to the recipient, and the potential for transmission of infectious agents are significant shortcomings of this approach. In some situations, such as replacement of blood vessels, vessels are removed from one portion of the body and grafted elsewhere to bypass sites of obstruction. However, the number of available vessels is

limited, and those available may not be optimal in terms of strength or other parameters.

Use of synthetic materials or tissues derived from animals offer alternatives to the use of human tissues. For example, grafts made of synthetic polymers such as Dacron find use in the replacement of vessels. Mechanical prostheses are widely used to replace damaged heart valves. However, use of synthetic materials has a number of disadvantages. Frequently the material is immunogenic and can serve as a nidus for infection or inflammation. Use of animal tissues also poses problems of immunogenicity as well as the potential to transmit diseases. In addition, harvested animal tissues may be suboptimal in terms of size, shape, or other properties, thus limiting the utility and flexibility of this approach. There is a need for innovative approaches to the problem of replacing damaged or dysfunctional organs and tissues.

Tissue engineering is an evolving field that seeks to develop techniques for culturing replacement tissues and organs in the laboratory (See, for example, Niklason, L. and Langer, R., Advances in tissue engineering of blood vessels and other tissues, *Transplant Immunology*, 5, 303-306, 1997, which is incorporated herein by reference). The general strategy for producing replacement tissues utilizes mammalian cells that are seeded onto an appropriate substrate for cell culture. The cells can be obtained from the intended recipient (e.g., from a biopsy), in which case they are often expanded in culture before being used to seed the substrate. Cells can also be obtained from other sources (e.g., established cell lines). After seeding, cell growth is generally continued in the laboratory and/or in the patient following implantation of the engineered tissue.

Tissue engineered constructs may be used for a variety of purposes including as prosthetic devices for the repair or replacement of damaged organs or tissues. They may also serve as *in vivo* delivery systems for proteins or other molecules secreted by the cells of the construct or as drug delivery systems in general. Tissue engineered constructs also find use as *in vitro* models of tissue function or as models for testing the effects of various treatments or pharmaceuticals.

Tissue engineering technology frequently involves selection of an appropriate culture substrate to sustain and promote tissue growth. In general, these substrates should be three-dimensional and should be processable to form scaffolds of a desired shape for the tissue of interest. Several classes of scaffolds are known. These scaffolds fall into five general categories: (1) non-degradable synthetic polymers; (2) degradable synthetic polymers; (3) non-human collagen gels, which are non-porous; (4) non-human collagen meshes, which are processed to a desired porosity; and (5) human (cadaveric) decellularized collagenous tissue. These different scaffold types are further discussed below.

Non-degradable synthetic polymers, e.g., Dacron and Teflon, may be processed into a variety of fibers and weaves. However, these materials are essentially non-biodegradable and thus represent a nidus for infection or inflammation following implantation into the body. Degradable synthetic polymers, including substances such as polyglycolic acid, polylactic acid, polyanhydrides, etc., may also be processed into various fibers and weaves and have been used extensively as tissue culture scaffolds. These materials may be modified chemically to "tune" their degradation rate and surface characteristics. However, fragments of degradable polyesters can trigger significant and undesirable inflammatory reactions.

Non-human collagen gels, e.g., gels made from bovine collagen and rat-tail collagen are convenient materials to work with in the laboratory, but suffer from significant drawbacks including poor tensile strength, no void volume to allow cell growth and tissue development, and sensitivity to collagenases that weaken the gels over time. Non-human collagen meshes consist of porous meshes made from processed bovine collagen. While the utility of these meshes for tissue engineering applications has been little studied, as with all materials made from bovine proteins they carry the risk of immunologic and/or inflammatory reactions when implanted into a human patient as well as the risk of contamination with agents of prion-based

disease.

In summary, none of the tissue culture scaffolds presently available is fully satisfactory from all points of view. Thus there exists a need for improved tissue culture scaffolds for use in tissue engineering.

In general, tissue culture scaffolds represent an intermediate in the production of tissue engineered products. The need for improved tissue culture scaffolds represents one aspect of the broader need for improved tissue engineered products for implantation into a human or animal to replace or supplement diseased, damaged, or absent tissues and/or organs. As in the case of animal tissues, tissue engineered tissues created using cells that are not obtained from the intended recipient may be antigenic. On the other hand, when using cells obtained from the intended recipient, a considerable period of time may be required to produce the tissue engineered tissue or organ, given that only a limited number of cells can be harvested. There is therefore a need for improved methods of producing tissue engineered tissues and organs with minimal antigenicity. There is also a need for more flexible methods of producing tissue engineered tissues and organs, for example, methods that would allow use of cells from the intended recipient while minimizing the time required to produce the engineered tissue or organ.

SUMMARY OF THE INVENTION

The present invention provides methods for producing scaffolds for use in tissue engineering and for producing tissue engineered constructs and engineered tissues for implantation into the body. The invention also provides scaffolds for use in tissue engineering, tissue engineered constructs, and engineered tissues suitable for implantation into the body based on the inventive methods. In addition, the invention provides methods for treating an individual in need of replacement or enhancement of a tissue or organ by implantation of the engineered constructs or tissues of the invention.

In one aspect, the invention provides methods for producing decellularized, tissue engineered constructs and also provides decellularized, tissue engineered constructs produced according to the inventive methods. In a preferred embodiment of the method a substrate is seeded (i.e., contacted) with a first population of cells,

preferably cells known to secrete extracellular matrix molecules such as collagen and elastin. The substrate can be flat, tubular, or, in general, can be configured to assume any desired three-dimensional shape. In a particularly preferred embodiment of the invention the substrate is tubular. Preferably the substrate consists of a biocompatible material, e.g., a biocompatible polymer having properties or incorporating modifications conducive to cell adherence and/or growth.

Appropriate cell types for seeding the substrate include fibroblasts and smooth muscle cells. In certain embodiments of the invention, the cells used to seed the substrate are derived from an individual of the same species as the individual into which the construct will ultimately be implanted in order to minimize immunogenicity. For example, if the construct is to be implanted into a human being, then human cells may be used to form the primary cell-seeded construct.

The construct is maintained in culture under conditions appropriate for growth of the cells for a growth period during which the cells secrete extracellular matrix molecules. In certain embodiments of the method multiple seedings and growth periods are employed. In certain embodiments of the invention more than one cell type is employed. For example, one or more seedings may be performed with a mixture of cells of different types. Alternatively, each seeding may employ cells of only one type but the same type is not necessarily used for all seedings. In certain embodiments of the invention growth conditions, e.g., tissue culture media, are selected to promote deposition of extracellular matrix. In certain embodiments of the invention stimuli, e.g., pulsatile forces, are applied to the construct during the growth period(s). Such stimuli may be selected to promote the development of desired properties such as mechanical strength.

After the cells have formed a tissue of the desired thickness, the construct is decellularized. Decellularization may be accomplished using any of a variety of detergents, emulsification agents, proteases, and/or high or low ionic strength solutions. In certain embodiments of the invention decellularization is performed under conditions and for sufficient times so that antigenic cells and cellular components are substantially removed, leaving a decellularized tissue engineered construct (scaffold) consisting primarily of extracellular matrix components such as collagen and elastin. In certain embodiments of the invention the substrate that was initially seeded is substantially or entirely removed from the scaffold.

Following the decellularization process, the decellularized construct may be washed to remove components of the decellularization solution. The decellularized construct may be subjected to additional tissue engineering steps as described below. In certain embodiments of the invention the decellularized construct is stored for later use. Different methods may be used to preserve the decellularized construct during storage including cryopreservation and drying according to a variety of protocols. Alternatively, the decellularized construct can be used immediately for further tissue engineering or implanted into the body of a subject. In certain embodiments of the invention the construct is treated with a biologically active agent prior to implantation.

In another aspect, the invention provides methods for producing engineered constructs suitable for implantation into the body. In certain embodiments of the invention, a decellularized tissue engineered construct is prepared from a tissue engineered construct as described above. The decellularized tissue engineered construct is implanted into the body and may recellularize *in vivo*. In certain embodiments of the invention, prior to implantation, the decellularized construct is treated with any of a variety of agents to enhance the recellularization process.

In an alternative method of the invention, prior to implantation into the body the decellularized tissue engineered construct is seeded with a population of cells to form a seeded decellularized tissue engineered construct. Before this seeding, the construct may be treated in various ways to enhance recellularization. The seeded construct may be implanted into the body of a subject (e.g., an animal, or preferably a human) in need thereof or may be maintained under conditions suitable for the growth and/or differentiation of the cells for a growth period prior to implantation.

In certain embodiments of the invention the cells employed for the seeding are derived from an individual of the same species as the individual into which the engineered construct is to be implanted. The cells may be derived from the same individual into which the engineered construct is to be implanted. A combination of different cell types can be used. For example, the decellularized tissue engineered construct can be seeded with a mixture of cells. Different cell types can be used to seed different portions or surfaces of the construct. In certain embodiments of the invention the cell type(s) are selected in accordance with the ultimate use of the engineered construct. For example, if the construct is to be used as an artery, then

appropriate cell types may include vascular cells such as endothelial cells, smooth muscle cells, and fibroblasts. If the construct is to be used to repair a cartilagenous structure, appropriate cell types may include chondrocytes and fibroblasts. In certain embodiments of the invention precursor cells are used to seed the decellularized culture scaffold. The precursor cells may differentiate during the second growth period and/or after implantation into an individual. In certain embodiments of the method multiple seedings and growth periods are employed. In certain embodiments of the invention more than one cell type is employed. For example, one or more seedings may be performed with a mixture of cells of different types. Alternatively, each seeding may employ cells of only one type but the same type is not necessarily used for all seedings. In certain embodiments of the invention stimuli, e.g., pulsatile forces, are applied to the construct during the growth period(s). Such stimuli may be selected to promote the development of desired properties such as mechanical strength.

In another aspect, the invention provides methods for producing decellularized, engineered native tissues and also provides decellularized, engineered native tissues produced according to the inventive methods. The method includes the steps of harvesting native tissue from an animal or human donor, subjecting the native tissue to one or more tissue engineering steps, and decellularizing the engineered native tissue. The tissue engineering step can comprise seeding the native tissue with cells and maintaining the seeded tissue for a growth period under conditions suitable for the growth of the cells. The tissue engineering step can comprise applying a mechanical or electrical stimulus to the native tissue, e.g., a pulsatile stimulus.

Following decellularization the tissue may be implanted into the body of a subject or subjected to further tissue engineering steps. Such steps may include any of the steps mentioned above, e.g., seeding with a population of cells and maintaining the seeded tissue for a growth period under conditions conducive to growth of the cells. The tissue may also be stored and subsequently retrieved for use. In those embodiments of the invention in which the decellularized, engineered native tissue is seeded with cells, the cells may be derived from the individual into whom the tissue is to be implanted. Seeding with cells from the individual into whom the tissue is to be implanted may decrease the likelihood of immune system rejection.

In another aspect, the invention provides methods of treating an individual in

need of replacement or enhancement of a tissue or organ. In certain embodiments, the methods comprise producing a decellularized tissue engineered construct or a decellularized engineered native tissue and implanting the construct or tissue into the body of the individual in accordance with standard surgical procedures. In certain embodiments, the methods comprise producing a decellularized tissue engineered construct or a decellularized engineered native tissue, seeding the construct or tissue with cells, and implanting the construct or tissue into the body of the individual in accordance with standard surgical procedures. In certain embodiments of the inventive method the construct or tissue is maintained in culture for a growth period under conditions conducive to growth of the seeded cells prior to implantation into the body of the individual. In certain embodiments of the invention the construct or tissue is seeded with cells that are derived from the individual. After implantation, cells from the individual may migrate into the tissue *in vivo*, complementing the seeded cell population. The migration of cells into the construct may be enhanced, e.g., by treating the construct with growth factors, chemotactic agents, or other compounds prior to or after implantation. The construct may include cells that are genetically engineered to produce one or more such growth factors, chemotactic agents, etc.

This application refers to various patents, articles, and other publications. The contents of all of these items are hereby incorporated by reference in their entirety.

DEFINITIONS

In order to more clearly and concisely point out the subject matter of the claimed invention, the following definitions are provided for specific terms used in the description and appended claims.

Allogeneic -- With respect to a recipient, an allogeneic cell or tissue is a cell or tissue that originates from or is derived from a donor of the same species as the recipient.

Animal -- As used herein, the term animal includes humans. Thus when referring to processes such as harvesting tissue from an animal, it is intended that the animal can be a human. Although at times reference will be made herein to "an animal or

human", this is not intended to imply that the term "animal" does not include a human.

Artificial substrate -- As used herein, the term artificial substrate includes materials such as degradable or non-degradable polymers synthesized *in vitro* (i.e., not produced by a living animal or plant). Note that the polymer may be identical to a polymer produced by a living plant or animal, e.g., the polymer may be a protein produced using recombinant DNA technology. The substrate can also be, for example, a length of tubing, which may be coated with any of a variety of artificial materials or materials obtained from natural sources. Artificial substrate also encompasses certain materials obtained by isolating and processing substances produced by a living source. In particular, the term encompasses materials obtained by harvesting tissue from an organism and isolating and/or processing one or more extracellular matrix proteins produced by a living source and therefore includes collagen sponges or rafts. However, a tissue that remains substantially intact and substantially retains the structure in which it is naturally found within the body of an organism is not considered an artificial substrate but is instead considered a native tissue. Other than this exception, the term "artificial substrate" is not intended to impose any limitation with respect to either material or configuration.

Autologous -- With respect to a recipient, an autologous cell or tissue is a cell or tissue that originates with or is derived from the recipient.

Biologically active agent -- A naturally occurring or synthetic chemical entity that is capable of inducing a change in the phenotype or genotype of a cell, tissue, organ, or organism when contacted with the cell, tissue, organ, or organism.

Cellular component -- This term refers to substances that constitute a portion of a cell, including cell membranes and macromolecules (e.g., nucleic acids or polypeptides) normally found enclosed within a cell membrane, embedded within a cell membrane, or attached to a cell membrane. The term does not include molecules that have been secreted by cells, e.g, extracellular matrix components such as collagen, elastin, and proteoglycans even if such molecules are linked to the cell surface.

Conditions suitable for growth -- Conditions suitable for growth of a particular cell type means an environment with conditions of temperature, pressure, humidity, nutrient and waste exchange, and gas exchange, that are permissive for the survival and reproduction of the cells. With respect to any particular type of cells, an environment suitable for growth may require the presence of particular nutrients or growth factors needed or conducive to the survival and/or reproduction of the cells.

Native tissue -- As used herein a native tissue is a tissue that is harvested from an animal or human and that remains substantially intact and substantially retains the structure in which it is naturally found within the body of the animal or human.

Non-cellular structural components -- As used herein a non-cellular structural component refers to a substance present within a biological tissue (either a native tissue or a tissue-engineered construct), the substance being derived from a cell that is or was present within the tissue but is not contained within the plasma membrane of a cell. Examples include collagen, elastin, proteoglycans, fibronectin, and laminin.

Precursor cell -- The term "precursor cell" refers to a cell that is not fully differentiated but that has the capacity to either become more fully differentiated itself or to give rise to a cell (or cells) that is able to further differentiate. The precursor cell may give rise to one or more different cell types. The process by which the precursor cell gives rise to a cell (or cells) that is able to further differentiate may involve one or more rounds of cell division. A stem cell is one type of progenitor cell. However, the term "progenitor cell" also includes cells that may have undertaken one or more steps along a differentiation pathway, e.g., that express one or more differentiation markers.

Primary cell-seeded construct -- A construct comprising an artificial substrate that has been seeded with a population of cells and maintained in culture under conditions suitable for growth and/or division of the cells for a period of time.

Secondary cell-seeded construct -- A primary cell-seeded construct that has been seeded with a second population of cells. The second population of cells may be

substantially equivalent to the population of cells that was used to produce the primary cell-seeded construct or may differ therefrom.

Tissue engineered construct -- This term is generally used herein to refer to a two or three dimensional mass of living mammalian tissue produced primarily by growth *in vitro*. The construct may include one or more types of tissue, and each tissue may include one or more types of cells. The term also encompasses a two or three dimensional mass of living mammalian tissue produced at least in part by growth *in vivo* on an artificial substrate. A tissue-engineered construct is distinguished from an explant of a corresponding natural tissue, e.g., a native tissue, in that the primary growth of the construct occurs *in vitro*.

Xenogeneic -- With respect to a recipient, a xenogeneic cell or tissue is a cell or tissue that originates from or is derived from a donor of a different species than the recipient.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows a bioreactor including a tubular substrate suitable for growth of a tissue engineered construct.

Figure 2A shows a photomicrograph of an untreated tissue engineered small caliber artery stained with hematoxylin and eosin at 66X magnification.
Figure 2B shows a photomicrograph of an untreated tissue engineered small caliber artery stained with hematoxylin and eosin at 100X magnification.

Figure 3A shows a photomicrograph of a decellularized tissue engineered small caliber artery stained with hematoxylin and eosin at 66X magnification.
Figure 3B shows a photomicrograph of a decellularized tissue engineered small caliber artery stained with hematoxylin and eosin at 100X magnification.

Figure 4A shows a photomicrograph of a decellularized tissue engineered small caliber bovine artery stained with hematoxylin and eosin at 66X magnification.

Figure 4B shows a photomicrograph of a decellularized tissue engineered small caliber bovine artery stained with hematoxylin and eosin at 100X magnification.

Figure 5A shows a photomicrograph of a tissue engineered small caliber porcine artery prior to decellularization, stained with hematoxylin and eosin.
Figure 5B shows a photomicrograph of a tissue engineered small caliber porcine artery following decellularization, stained with hematoxylin and eosin.

Figure 6A shows a phase contrast view of a cross section of a seeded decellularized porcine artery cross section.
Figure 6B shows a fluorescent cross section of the same sample shown in Figure 6B.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

The invention provides a variety of new methods and products of utility in the field of tissue engineering and replacement tissues and organs. More specifically, in one aspect the invention provides methods for producing tissue engineered constructs that can be implanted into the body, e.g., as treatments for conditions involving tissue damage or dysfunction. As used herein, implanting into the body includes implanting on the body surface and/or attaching onto the body in addition to implanting within the body so that the implanted construct or tissue is entirely enclosed within the body. Thus the constructs and tissues of the present invention can include replacements for skin, cornea, and other tissues that are not strictly within the body.

In general, the constructs of the present invention are produced by first growing a tissue engineered construct according to any of a variety of methods as described below. Typically these methods involve seeding (i.e., contacting) a substrate with cells and culturing the seeded substrate under conditions suitable for growth of the cells to form a tissue engineered construct. As the cells grow and divide on and/or in the substrate they secrete extracellular matrix proteins such as collagen and elastin. The construct is cultured for a period of time sufficient to produce a construct of desired thickness and/or properties, consisting primarily of secreted proteins and cells. Various growth conditions can be selected to enhance this process and/or to stimulate the development of desirable mechanical, physical, or

biochemical properties, etc. Such growth conditions may include the use of particular growth media, the application of mechanical, electrical, and/or chemical stimuli, etc. The cells may be derived from an animal or cell line of the same species as the intended recipient, so that the resulting construct contains proteins that will be minimally antigenic and maximally compatible in the body. For example, if the construct is to be implanted into a human, the cells may be human cells. Although in general production of the tissue engineered construct involves culturing the developing tissue primarily *in vitro*, tissue engineered constructs produced at least in part by culturing the tissue *in vivo* are also within the scope of the invention.

In certain embodiments of the invention, production of the tissue engineered construct involves multiple rounds of cell seeding and intervening growth periods. The cells used in different seedings may be of the same or different types and/or may consist of multiple cell types. The growth periods and culture conditions may be the same or may vary between different growth periods. For example, in certain embodiments of the invention, to produce a tissue engineered blood vessel, a substrate is seeded with smooth muscle cells and cultured for a period of time, e.g., 6 weeks. After this first growth period the construct is seeded with endothelial cells and cultured for a further growth period, e.g., 1-2 weeks.

Following production of the tissue engineered construct, regardless of the particular steps employed in its production, the construct is decellularized. Appropriate decellularization techniques remove cellular components while leaving the secreted proteins, e.g., collagen and elastin, substantially intact. Thus, one method of the present invention includes the steps of (i) producing a tissue engineered construct by seeding a substrate with cells, allowing the cells to grow in culture, and optionally subjecting the construct to one or more additional rounds of cell seeding and growth; and (ii) decellularizing the tissue engineered construct, thereby producing a decellularized construct. The first step can include performing various tissue engineering manipulations such as applying mechanical or electrical stimuli to the developing construct, applying selected biologically active agents to the construct (e.g., growth factors). In certain embodiments of the invention the decellularized construct retains substantially the same shape and physical properties as prior to decellularization. In particular, connective tissues such as blood vessels, muscle, bone, tendon, and ligament, all of which have substantial components of extracellular

matrix proteins, derive most of their mechanical strength from their extracellular matrix components. The contribution of cells to the physical characteristics of connective tissues is rather small. Thus treatments that remove cells while causing little damage to the extracellular matrix are preferable.

In preferred embodiments of the invention the decellularized construct is thoroughly washed to remove residual decellularization solution that may reduce biocompatibility or inhibit subsequent growth of cells on or in the construct. In certain embodiments of the invention the decellularization process and/or subsequent washing steps result in removal of most or substantially all of the substrate (i.e., the material on which the cells were initially seeded) that remains after the growth period. Following decellularization, the construct can be implanted into the body of a subject or stored before further use. In the latter case, when a patient is in need of an implanted tissue, the construct can readily be reconstituted. Such reconstitution may include the removal of residual storage solution, etc. Thus the inventive method includes one or more of the optional steps of (i) washing the decellularized construct; (ii) removing some or all of the remaining substrate; (iii) storing the decellularized construct; (iv) reconstituting the decellularized construct; and (v) implanting the decellularized construct into a subject.

In contrast to tissues harvested from animal or human donors, the substrate (and therefore the decellularized construct itself) can be configured to assume a particular desired shape and size without the constraints that are imposed by the shape or size of harvested tissue. For example, engineered blood vessels can be grown to a certain desired length or diameter without undesired structures such as side branches or valves. Also, in contrast to decellularized animal tissues, the cell-derived proteins in the decellularized construct can come from cells of the same species as the intended recipient. While not wishing to be bound by any theory, human extracellular matrix proteins are expected to be essentially non-immunogenic when implanted into a human, which may make them preferable to proteins derived from animal sources and also preferable to synthetic degradable or non-degradable polymers. This is so because the extracellular matrix components that make up the non-cellular structural components of a decellularized construct are highly conserved within a species. For example, genetic variants in collagens and elastins are quite rare. Furthermore, cells (e.g., fibroblasts or smooth muscle cells) can be obtained from a single donor and

used to produce large numbers (e.g., hundreds) of constructs. These cells may be rigorously screened for transmissible diseases (e.g., HIV or hepatitis), thus decreasing the infectious risk associated with the products. Engineered tissues can be produced using cells that have desirable properties such as an ability to grow well in culture, that have been genetically modified to alter, for example, their secretion of extracellular matrix components, etc.

In certain embodiments of the invention the decellularized construct is used as a scaffold for further tissue engineering. In the case that the scaffold was stored after the decellularization process, the scaffold may be reconstituted as appropriate depending upon the storage technique employed. The decellularized construct, also referred to herein as a scaffold, is seeded with a population of cells, which may be substantially equivalent to the population of cells that was used to seed the substrate or may be different in one or more respects. For example, the cells used to seed the scaffold may be of a different cell type or species from the cells that were used to produce the decellularized construct. In general, the cells are of the same species as the intended recipient and are of a cell type characteristic of the tissue or organ that the construct is intended to replace or augment. For example, if the construct is a blood vessel, the cells preferably include endothelial cells and smooth muscle cells. As in the case of initial production of the tissue engineered construct, multiple rounds of cell seeding and intervening growth periods can be employed. The cells used in different seedings may be of the same or different types and/or may consist of multiple cell types. The growth periods and culture conditions may be the same or may vary between different growth periods. Various growth conditions can be selected to enhance this process and/or to stimulate the development of desirable mechanical, physical, or biochemical properties, to stimulate migration of cells into the wall of the construct, etc. Such growth conditions may include the use of particular growth media, the application of mechanical, electrical, and/or chemical stimuli, etc. Thus, in general, the decellularized construct (scaffold) may be subjected to any of the tissue engineering steps involved in production of a tissue engineered construct.

In certain embodiments of the invention the decellularized construct is seeded with cells obtained from the individual who is the intended recipient of the construct. This approach minimizes the likelihood that the construct will cause an

immunological or inflammatory reaction when implanted into the recipient. This embodiment of the invention represents an especially advantageous strategy for the production of a cell-based implantable tissue. For example, using current techniques it takes approximately 6 -10 weeks of culture time to produce an implantable tissue engineered artery from cells that are seeded and grown on degradable polymer scaffolds (Niklason, *et al.*, Functional arteries grown in vitro, *Science*, 284: 489-93,1999). The availability of decellularized and mechanically robust collagenous scaffolds as provided by the present invention dramatically shortens this production time. According to one embodiment of the inventive methods, when a patient who would benefit from an implantable vessel is identified, a small biopsy is taken from the patient and the cells isolated. The cells are then seeded onto a decellularized scaffold and grown in culture for a period of several days to one or two weeks. Then the complete, essentially autologous vessel is implanted. This approach reduces the total culture time to produce an autologous vessel from 6 -10 weeks to 1-2 weeks, a reduction with profound implications from the point of view of clinical applicability. Of course the methods have similar benefit with respect to other implantable tissues, e.g., heart valves, bladders, etc.

The scaffold may be treated in any of a variety of ways either before or after seeding. For example, agents selected to enhance the adherence or growth of the cells may be applied to the scaffold. After seeding, the seeded scaffold may be implanted into a subject or may be cultured for one or more additional growth periods (i.e., in addition to the period(s) of growth prior to decellularization). In the latter case, various growth conditions can be selected to enhance cell growth and division and/or to stimulate the development of desirable mechanical, physical, or biochemical properties, to stimulate migration of cells into the wall of the scaffold, etc. Such growth conditions may include the use of particular growth media, the application of mechanical, electrical, and/or chemical stimuli, etc.

Thus in summary the inventive methods optionally include the additional steps of (i) seeding the scaffold (i.e., the decellularized construct) with a population of cells, thereby obtaining a cell-seeded decellularized construct; and (ii) implanting the cell-seeded decellularized construct into a subject. Prior to the second of these steps the construct may be maintained in culture for a period of time under conditions suitable for growth and/or division of the cells, thereby producing a tissue engineered

decellularized construct. As in the case of production of the initial tissue engineered construct, the tissue engineered, recellularized, decellularized construct may be subjected to multiple rounds of cell seeding and growth, each of which may involve different cell type(s) and/or different growth conditions. During one or more growth periods the tissue engineered decellularized construct may be subjected to various tissue engineering manipulations such as the application of mechanical or electrical stimuli.

In general, the constructs of the present invention can be treated with any of a variety of biologically active agents prior to implantation into a subject. In certain embodiments of the invention these agent(s) are selected to enhance the properties of the construct following implantation, e.g., to facilitate the ability of endogenous cells (i.e., cells present within the subject) to populate the construct, to enhance the growth of seeded cells, to facilitate vascularization of the construct, to reduce the likelihood of thrombus formation, etc. Appropriate biologically agents include, but are not limited to, thrombomodulators, agents that increase hemocompatibility, and antibiotics. In certain embodiments of the invention the biologically active agent comprises a pharmaceutical composition. In this case the construct may serve as a drug delivery vehicle. The pharmaceutical composition may be intended for treatment of the same condition as that being treated by implanting the construct or for treatment of a different condition.

In addition to the decellularization of constructs obtained through tissue engineering techniques (e.g., constructs obtained by seeding an artificial substrate), the present invention also encompasses the decellularization of native tissue that has been subjected to certain tissue engineering step(s) prior to decellularization. For example, the native tissue may be cultured for a period of time under conditions suitable for growth and division of the cells contained therein after harvesting. The native tissue may be seeded with additional cells. The growth conditions (e.g., the growth medium) may be selected to enhance cell growth and division and/or to stimulate the development of desirable mechanical, physical, or biochemical properties. For example, mechanical or electrical forces may be applied to the native tissue. Following the tissue engineering steps the native tissue is decellularized and may then be stored, implanted into the body of a subject, or used as a scaffold for further tissue engineering. In the latter case, the decellularized native tissue is seeded

with a population of cells and may then be used or further processed in essentially the same manner as the decellularized constructs described above.

In the following sections, techniques and conditions for production of a primary cell-seeded construct, techniques for decellularization of the primary cell-seeded construct to produce a decellularized construct (scaffold), methods for storage of the scaffold, and methods for reconstitution of the scaffold after storage are described in further detail. Methods for using the scaffold to produce a tissue engineered construct for implanting into the body are also described in more detail below. In addition, tissue engineering steps that may be applied to a harvested native tissue prior to decellularization are described.

In certain embodiments of the invention the construct to be decellularized comprises a tissue engineered construct produced as described in the pending patent application entitled, "Tissue-Engineered Constructs", Ser. No. 09/109,427, filed 07/02/98.

Production of a Tissue Engineered Construct

Numerous methods and techniques for producing tissue engineered constructs are known in the art and are appropriate for use in conjunction with the present inventive methods. Examples of suitable seeding and culturing methods for the growth of three-dimensional cell cultures are disclosed in pending application "Tissue-Engineered Constructs" Ser. No. 09/109,427; U.S. Pat. No. 5,266,480, and U.S. Patent No. 5,770,417, all three of which are incorporated herein by reference. These references disclose techniques for establishing a three-dimensional matrix, inoculating the matrix with the desired cells, and maintaining the culture. In general, a tissue engineered construct is produced by seeding cells onto an appropriate substrate and culturing the cells under conditions suitable for growth. The substrate can be flat, tubular, or, in general, can be configured to assume any desired three-dimensional shape. For example, the substrate may be formed into shapes including but not limited to spheres, ellipsoids, disks, sheets, or films as well as hollow spheres, hollow ellipsoids, and open-ended, hollow tubes. In certain embodiments of the invention the substrate is tubular.

In certain embodiments of the invention the substrate comprises a biocompatible material, e.g., a biocompatible polymer having properties or

incorporating modifications conducive to cell adherence and/or growth. Suitable materials include materials that are biodegradable or bioerodable, such as materials that hydrolyze slowly under physiological conditions. Porous materials are preferred in certain embodiments of the invention. Among the various suitable materials are synthetic polymeric materials such as polyesters, polyorthoesters, or polyanhydrides, including polymers or copolymers of glycolic acid, lactic acid, or sebacic acid. Substrates comprising proteinaceous polymers are also suitable for production of tissue engineered constructs. Collagen gels, collagen sponges and meshes, and substrates based on elastin, fibronectin, laminin, or other extracellular matrix or fibrillar proteins may be employed. Either synthetic polymers or proteinaceous polymers may be modified or derivatized in any of a variety of ways, e.g., to increase their hydrophilicity and/or provide improved cell adhesion characteristics. In certain embodiments of the invention the substrate is coated with a material, e.g., denatured collagen, prior to seeding in order to increase adherence of the cells thereto. Materials useful as substrates for growing cells to produce tissue engineered substrates, and methods of producing such substrates are known in the art and are described in pending application "Tissue-Engineered Constructs", Ser. No. 09/109,427, and in U.S. Patent No. 5,770,417.

In certain embodiments of the invention some or all of the substrate degrades during the growth period and/or is removed prior to implantation of the construct into a subject. Removal may be accomplished by application of a fluid flow and may be enhanced by decellularization. In certain embodiments of the invention a tissue engineered construct is grown on a structure from which it is completely removed after a growth period. For example, a vascular construct may be grown on a length of silicone tubing that has been coated with a thin layer of dilute, denatured human collagen to which cells can adhere.. After the growth period the silicone tubing is removed from the vascular construct, resulting in a tissue engineered construct entirely free of substrate.

A number of different cell types or combinations thereof may be employed in the present invention, depending upon the intended function of the tissue engineered construct being produced. These cell types include, but are not limited to: smooth muscle cells, cardiac muscle cells, epithelial cells, endothelial cells, urothelial cells, fibroblasts, myoblasts, chondrocytes, chondroblasts, osteoblasts, osteoclasts,

keratinocytes, hepatocytes, bile duct cells, pancreatic islet cells, thyroid, parathyroid, adrenal, hypothalamic, pituitary, ovarian, testicular, salivary gland cells, adipocytes, and precursor cells. For example, smooth muscle cells and endothelial cells may be employed for muscular, tubular constructs, e.g., constructs intended as vascular, esophageal, intestinal, rectal, or ureteral constructs; chondrocytes may be employed in cartilaginous constructs; cardiac muscle cells may be employed in heart constructs; hepatocytes and bile duct cells may be employed in liver constructs; epithelial, endothelial, fibroblast, and nerve cells may be employed in constructs intended to function as replacements or enhancements for any of the wide variety of tissue types that contain these cells. In general, any cells may be employed that are found in the natural tissue to which the construct is intended to correspond. However, in some cases it may be advantageous to employ cells of a type that is not naturally found in the tissue to which the construct is intended to correspond. In addition, progenitor cells, such as myoblasts or stem cells, may be employed to produce their corresponding differentiated cell types. In some instances it may be preferred to use neonatal cells or tumor cells.

In certain embodiments of the invention the cells are allogeneic to the intended recipient rather than xenogeneic. Cells may be obtained from a donor (either living or cadaveric) or derived from an established cell line. To obtain cells from a donor (e.g., a potential recipient of a tissue engineered construct), standard biopsy techniques known in the art may be employed. Representative techniques are described, for example, in pending application "Tissue-Engineered Constructs", Ser. No. 09/109,427 and in Oberpenning, F., *et al.*, De novo reconstitution of a functional mammalian urinary bladder by tissue engineering, *Nature Biotechnology*, 17, 149-155, 1999. The contents of this article, which also describes appropriate materials and techniques for creation of three-dimensional substrates, cell culture and cell seeding techniques, and methods for evaluation of tissue engineered organs, are incorporated herein by reference. Cells so obtained may be expanded in culture, although preferably cells of a low passage number (e.g., less than 5 or, more preferably, less than 3) are used to produce the construct in order to minimize loss of the differentiated phenotype. Preferably cells isolated from a donor are screened to eliminate the potential for transmission of infectious diseases. Cells derived from established cell lines (e.g., those available from the ATCC, Rockville, MD) may also be used. In certain

embodiments of the invention cells (either obtained from a donor or from an established cell line) that have been genetically manipulated by the introduction of exogenous genetic sequences or the inactivation or modification of endogenous sequences are employed. For example, genes may be introduced to cause the cells to make proteins that are otherwise lacking in the host. Production of scarce but desirable proteins (in the context of certain tissues) such as elastin may be enhanced.

As mentioned above, in order to minimize antigenicity in certain embodiments of the invention cells from the same species as the intended recipient of the final construct are employed to create the initial tissue engineered construct (i.e., the construct that is to be decellularized). Thus if the construct is to be implanted into a human, preferably cells derived from a human are used to create the initial construct. In those embodiments of the invention in which the decellularized construct is employed as a scaffold for further tissue engineering (i.e., those embodiments in which the decellularized construct is seeded with cells), cells from the same species as the intended recipient of the final construct are preferably used to seed the decellularized construct. In certain embodiments of the invention the decellularized construct is seeded with cells harvested from the intended recipient of the construct. General mammalian cell culture techniques, cell lines, and cell culture systems that may be used in conjunction with the present invention are described in Doyle, A., Griffiths, J.B., Newell, D.G., (eds.) *Cell and Tissue Culture: Laboratory Procedures*, Wiley, 1998, the contents of which are incorporated herein by reference.

In certain embodiments of the invention mammalian cells are seeded onto and/or within a substrate from a suspension so that, preferably, they are evenly distributed at a relatively high surface and/or volume density. The substrate may be, but need not be, a porous substrate. The cell suspensions may comprise approximately 1 X 10^4 to 5 X 10^7 cells/ml of culture medium, more preferably approximately 2 X 10^6 cells/ml to 2 X 10^7 cells/ml, and yet more preferably approximately 5 X 10^6 cells/ml. The optimal concentration and absolute number of cells may vary with cell type, growth rate of the cells, substrate material, and a variety of other parameters. The suspension may be formed in any physiologically acceptable fluid, preferably one that does not damage the cells or impair their ability to adhere to the substrate. Appropriate fluids include standard cell growth media (e.g., DMEM with 10% FBS).

The cells may be seeded onto and/or within a substrate by any standard method. For example, the substrate may be seeded by immersion in a cell suspension for a period of time during which cells adhere to the substrate, followed by washing away the nonadherent cells. The substrate may be seeded with cells using a syringe, pipet, or other sterile delivery apparatus. According to a preferred method the cell suspension is dripped onto the substrate, and the substrate is subsequently rotated, e.g., in a rotating vessel to promote even distribution of the cells.

Following seeding of the cells, in certain embodiments of the invention the cells are allowed to adhere to the substrate for a period of time (seeding time) prior to placing the seeded substrate in tissue culture medium. The optimum seeding time varies with cell type and substrate. For example, when using the synthetic hydrophilic polymeric substrates disclosed in pending application "Tissue-Engineered Constructs", Ser. No. 09/109,427, seeding times of approximately 20 minutes may be used. For other substrates, seeding times of an hour or more may be appropriate and have been employed in the prior art.

Various treatments may be applied to enhance adherence of cells to the substrate and/or to each other. Appropriate treatments are described, for example, in the above-mentioned pending application and in U.S. Patent No. 5,613,982. Such treatments include the application of various proteins, e.g., growth factors or extracellular matrix proteins to the substrate or to the growing construct. For example, collagen, elastin, fibronectin, laminin, or proteoglycans may be applied to the substrate. The substrate can be impregnated with growth factors such as aFGF, bFGF, PDGF, TGFβ, VEGF, etc., or these agents may be provided in the culture medium.

Appropriate growth conditions for mammalian cells in culture are well known in the art. Cell culture media generally include essential nutrients and, optionally, additional elements such as growth factors, salts, minerals, vitamins, etc., that may be selected according to the cell type(s) being cultured. Particular ingredients may be selected to enhance cell growth, differentiation, secretion of specific proteins, etc. In general, standard growth media include Dulbecco's Modified Eagle Medium, low glucose (DMEM), with 110 mg/L pyruvate and glutamine, supplemented with 10-20% fetal bovine serum (FBS) or calf serum and 100 U/ml penicillin are appropriate as are various other standard media well known to those in the art. A particularly

preferred culture medium for producing a muscular, tubular tissue engineered construct such as a small caliber blood vessel is described in Example 2 below. Preferably cells are cultured under sterile conditions in an atmosphere of 5-15% CO_2, preferably 10% CO_2, at a temperature at or near the body temperature of the animal of origin of the cell. For example, human cells are preferably cultured at approximately 37°C.

In general, the length of the growth period will depend on the particular tissue engineered construct being produced. The growth period can be continued until the construct has attained desired properties, e.g., until the construct has reached a particular thickness, size, strength, composition of proteinaceous components, and/or a particular cell density. Methods for assessing these parameters are described in pending application "Tissue-Engineered Constructs", Ser. No. 09/109,427, and in U.S. Patent No. 5,613,982.

Following a first growth period the construct can be seeded with a second population of cells, which may comprise cells of the same type as used in the first seeding or cells of a different type. The construct can then be maintained for a second growth period which may be different in length from the first growth period and may employ different growth conditions. Multiple rounds of cell seeding with intervening growth periods may be employed.

In certain embodiments of the invention a muscular, tubular tissue engineered construct is grown in a biomimetic system such as that described in pending application "Tissue-Engineered Constructs", Ser. No. 09/109,427 and in Niklason, *et al.*, Functional arteries grown in vitro, *Science*, 284: 489-93,1999. As described therein, a semi-disposable glass bioreactor similar to that shown in Figure 1 and discussed in Example 1 of the present application is attached to a pump system. As shown in Figure 1, the bioreactor chamber 22 includes side arms 12 through which a length of tubing 14 is inserted. The tubing serves as a support for a substrate 16 which is seeded to produce the construct. Alternately, the tubing itself may serve as a substrate either with or without a layer or layers of coating material. Fluid can be pumped through the tubing to impart a pulsatile force to the lumen of the developing construct as discussed below. The bioreactor includes a stopper 18 that can be removed to place the substrate within the reactor and to seed the substrate with cells. Culture medium and other fluids are added to and removed from the chamber via a

medium fill port 20. The bioreactor system may be made of glass or of another appropriate material such as various plastics. In those embodiments of the invention in which the decellularized construct is cryopreserved, the bioreactor is preferably made of a material such as plastic capable of withstanding extremely low temperatures (e.g., that of liquid nitrogen).

Application of stimuli during growth period

Tissues within the body are subjected to a variety of physical stimuli. For example, arteries, heart valves, and heart chambers are exposed to pulsatile stretch and flow forces as blood is pumped through the cardiovascular system. Components of the musculoskeletal system are subjected to mechanical forces during walking and other physical activities. It is well established that physical stimuli can exert profound effects on the properties and development of tissues and of the cells that produce these tissues. Without wishing to be bound by any theory, we propose that exposing developing tissue engineered constructs to certain stimuli (e.g., mechanical forces that resemble those to which corresponding tissues would normally be exposed in vivo) will cause the resulting construct to develop properties and structure that more closely resemble those of the corresponding naturally occurring tissue. In some instances the application of appropriate stimuli may result in desirable properties, e.g., increased strength, that exceed those found in the naturally occurring tissue. Therefore, in certain embodiments of the invention a physical stimulus (e.g., a mechanical or electrical stimulus) is applied to the tissue engineered construct during the growth periods. The strength and nature of the stimulus may be varied during the growth period, and the stimulus need not be applied continuously throughout the growth period but may be applied during one or more portions of the growth period. In the case of a construct that is produced by performing multiple rounds of cell seeding with intervening growth periods, different stimuli may be employed during different growth periods.

In certain embodiments of the present invention, as described in detail in pending application "Tissue-Engineered Constructs" Ser. No. 09/109,427, a muscular, tissue engineered construct is produced in which a distensible body is inserted within the lumen of a substrate to provide pulsatile stretch to seeded muscle cells. While the muscle tissue is growing on and/or within the substrate, a pump in communication

with the interior of the distensible body provides cyclic increases in pressure to cause the distensible body to distend within the lumen of the substrate and impart a pulsatile stretching force to the substrate and the developing tissue. The application of pulsatile stretching forces may be used in the production of both vascular tissue engineered constructs and muscular, nonvascular constructs such as esophageal, intestinal, rectal, ureteral, or bladder constructs. The forces applied to the construct may be selected to mimic corresponding natural forces in terms of pulsation and the degree of stretch imparted to the construct.

In certain embodiments of the invention forces are applied to a muscular, tubular tissue engineered construct without the use of a distensible tube. For example, fluid such as tissue culture medium can be pumped directly through the lumen of the construct, thus mimicking intraluminal flow as found in arteries in the body. The flow may be varied as the construct develops, and the intraluminal pressure and shear forces may even be increased beyond those found in the body.

Of course the application of physical forces is not limited to muscular and/or tubular tissue engineered constructs but may be advantageously employed in the production of a variety of other types of tissue engineered constructs. For example, pulsatile flow can be employed in the production of heart valves as described in U.S. Patent No. 5,899,937, the contents of which are herein incorporated by reference. The application of stimuli is not limited to the application of pulsatile stimuli, stretching forces, or stimuli related to fluid flow. For example, compressive stimuli, either constant or cyclical may be employed. In addition, non-mechanical stimuli such as electrical stimuli may be employed.

Decellularization

The methods discussed in this section may be applied to a tissue engineered construct produced according to any of the methods described above or to a native tissue that has been harvested from a subject. In the former case, the result of decellularizing is to produce a decellularized, tissue engineered construct. The decellularized, tissue engineered construct can be implanted into a subject, subjected to further tissue engineering steps that may include seeding with cells, or used for other purposes. A native tissue can be subjected to tissue engineering steps before decellularization to produce an engineered, decellularized native tissue. Of course the

engineered, decellularized native tissue can be subjected to additional tissue engineering steps after decellularization.

Decellularization has a number of effects. In particular, in the case of a tissue engineered construct that is produced using cells that are allogeneic to an intended recipient (i.e., cells that are derived from the same species as the recipient), the extracellular matrix proteins such as collagen and elastin that make up a large portion of the construct are substantially non-immunogenic when implanted into the recipient. However, the cells themselves are generally immunogenic when implanted into a subject other than the individual from whom the cells were derived (or a genetically identical individual). For example, pure human collagen (either obtained from human tissue or produced using recombinant DNA technology) is generally non-immunogenic when implanted into a human subject. However, human cells that produce collagen are generally immunogenic when implanted into a human being other than the individual from which they were derived. In other words, in a typical tissue engineered construct the cells constitute the majority of the antigenic material in the construct. Therefore, by removing the cells, it is possible to substantially reduce or eliminate the likelihood that an immunologic or inflammatory reaction will be induced upon implanting the construct into a subject.

Any of a number of decellularization methods can be employed. In general the methods employ a variety of chemical, biochemical, and/or physical means to disrupt, degrade, and/or destroy cellular components and/or modify the matrix in which the cells are embedded so as to facilitate removal of the cells and cellular components. Such methods are disclosed, for example, in U.S. Pat. No. 4,776,853, U.S. Pat. No. 5,192,312, U.S. Pat. No. 5,336,616, U.S. Pat. No. 5,595,571, U.S. Pat. No. 5,613, 982, U.S. Pat. No. 5,855,620, U.S. Pat. No. 5,899,936, and U.S. Pat. No. 5,916,265. The disclosures of these eight patents are incorporated herein by reference. Additional decellularization methods are disclosed in Bader, A., et al., Tissue engineering of heart valves - human endothelial cell seeding of detergent acellularized porcine valves, *Eur. J. Cardio-thoracic Surg.*, 14, 279-284, 1998 and in Courtman, D.W., et al., Biomechanical and ultrastructural comparison of cryopreservation and a novel cellular extraction of porcine aortic valve leaflets, *J. Biomed. Mat. Res.*, 29, 1507-1516, 1996. The contents of these two articles are incorporated herein by reference. Of course the invention is not limited to these

decellularization techniques but also includes modifications of these techniques, as well as other techniques currently available or developed in the future.

The decellularization method preferably does not cause gross alteration in the structure of the tissue engineered construct or native tissue or cause substantial alteration in its biomechanical properties. The effects of decellularization on structure may be evaluated by light microscopy, ultrastructural examination, etc. Biomechanical tests, which are well known in the art, may be used to evaluate the effects of various decellularization protocols on tissue properties. Selection and interpretation of such tests will depend, in general, upon the nature of the construct and the purpose for which it is intended. In addition, the treatment preferably does not result in a cytotoxic environment that significantly inhibits subsequent steps such as reseeding *in vitro* or population of the construct or tissue by cells of a recipient *in vivo*.

In certain embodiments of the invention the construct or tissue to be decellularized is incubated in one or more decellularization solutions for a period of time sufficient to remove a substantial fraction of the cells and/or cellular components. In general, the decellularization solutions enhance cell lysis and destruction of cellular components, e.g., they contain agents that disrupt and/or degrade cellular constituents such as cell membranes, proteins, nucleic acids, etc. Aqueous hypotonic or low ionic strength solutions facilitate cell lysis through osmotic effects. Such solutions may comprise deionized water or an aqueous hypotonic buffer (e.g., at a pH of approximately 5.5 to 8, preferably approximately 7 to 7.5). Decellularization may be accomplished using a single decellularization solution, or the construct may be incubated sequentially in two or more solutions. Another approach involves immersing the construct in alternating hypertonic and hypotonic solutions.

Suitable decellularization agents include salts, detergent/emulsification agents and enzymes such as proteases, and/or nucleases. Combinations of different classes of detergents, e.g., a nonionic detergent such as Triton X-100® (tert-octylphenylpolyoxyethylene) and an ionic detergent such as SDS (sodium dodecyl sulfate) may be employed. In a particularly preferred embodiment of the inventive method, one or more decellularization solutions that include Triton X-100®, CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), or SDS in

phosphate buffered saline (PBS) is employed as described in the Examples below. Other suitable detergents include polyoxyethylene (20) sorbitan mono-oleate and polyoxyethylene (80) sorbitan mono-oleate (Tween 20 and 80), sodium deoxycholate, and octyl-glucoside.

In general, it is preferable to employ a decellularization technique that minimizes damage to or alteration of the proteinaceous matrix. Such damage may result from proteases (e.g., collagenase) that may be released upon lysis of cells or that may be present in the matrix extracellularly. Therefore, in certain embodiments of the invention various additives such as metal ion chelators, e.g., EDTA (ethylenediaminetetraacetic acid) and/or protease inhibitors are included in the decellularization solution. Suitable protease inhibitors for use in decellularization solutions include, for example, one or more of the following: phenylmethylsulfonyl-fluoride (PMSF), aprotinin, leupeptin, and N-ethylmaleimide (NEM).

Various enzymes that degrade cellular components may be employed in the decellularization solution. Such enzymes include nucleases (e.g., DNAses such as DNAse I, RNAses such as RNAse A), and phospholipases (e.g., phospholipase A or C). Certain proteases such as dispase II, trypsin, and thermolysin may be of use in decellularization, particularly in decellularization of native tissues such as skin. When employing proteolytic enzymes it may be desirable to take care that removal of cells occurs without significant damage to the extracellular matrix. The activity of proteases is a function of time, temperature, and concentration, and these variables may be appropriately adjusted to achieve acceptable decellularization without unacceptable destruction of the extracellular matrix. Nucleases are typically employed at a concentration of between 0.1 μg/ml and 50 μg/ml, preferably approximately 10 μg/ml for DNAse I and approximately 1.0 μg/ml for RNAse A. The nucleases are preferably employed in a physiologically buffered solution at a temperature of between approximately 20°C to 38°C, preferably 37°C, for a time between approximately 30 minutes to 6 hours.

As mentioned above, the decellularization solution typically includes a buffer. Suitable buffers include organic buffers such as Tris (hydroxymethyl)aminomethane (TRIS), (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), etc. Buffers including sodium phosphate, citrate, bicarbonate, acetate, or glutamate may also be used. In general, a pH between about 5.5 and 8.0, between about 6.0 and 7.8,

or between about 7.0 and 7.5 is employed.

Physical forces such as the formation of intracellular ice may be employed as a primary means of accomplishing decellularization or to augment the activity of decellularization solutions. One such approach referred to as vapor phase freezing involves placing the construct or tissue in an appropriate solution, e.g., a standard cryopreservation solution such as Dulbecco□s Modified Eagle Medium (DMEM), 10% dimethylsulfoxide (DMSO), 10% fetal bovine serum (FBS) and cooling at a slow rate, e.g., 1-2°C/min. Multiple freeze-thaw cycles may be employed. Colloid-forming materials may be added to the solution to reduce extracellular ice formation while allowing formation of intracellular ice. Appropriate materials include polyvinylpyrrolidone (10% w/v) and dialyzed hydroxyethyl starch (10% w/v).

The examples of decellularization techniques provided above are not intended to be limiting, and the invention encompasses the use of essentially any decellularization technique that removes a substantial fraction of the cells while leaving the matrix substantially intact. Of course it is to be understood that certain techniques will be preferred for particular tissue engineered constructs or native tissues, depending upon the properties of these constructs or tissues. One of ordinary skill in the art will be able to select an appropriate decellularization technique and to vary parameters such as temperature and time in order to achieve a desired degree of decellularization. In certain embodiments of the invention the decellularization process removes at least 50% of the cells. In certain embodiments of the invention the decellularization process removes at least 60%, at least 70%, or at least 80% of the cells. In certain embodiments of the invention at least 90%, at least 95%, or substantially all of the cells are removed. As described above, there may be a tradeoff between the two goals of achieving a high degree of decellularization and preserving the structure and properties of the extracellular matrix. Thus it is not necessarily preferred to achieve maximal possible decellularization if doing so results in unacceptable damage to the extracellular matrix. The optimum degree of decellularization may depend upon the properties of the construct and the use for which it is intended.

Regardless of the decellularization method employed, in certain embodiments of the inventive methods the decellularized construct or tissue is washed in a physiologically appropriate solution such as PBS, tissue culture medium, etc.,

following removal from the solution in which decellularization was performed. Washing removes residual decellularization solution that might otherwise cause deterioration of the decellularized construct or tissue, inhibit the growth of subsequently seeded cells, and/or reduce biocompatibility.

In certain embodiments of the invention decellularization is performed by soaking the construct in decellularization solution(s) for a period of time. The solution may be stirred or agitated during this period. In addition, it may be desirable to alter the pattern of flow of the decellularization solution, e.g., by establishing convective currents within the container in which decellularization is performed, by employing rotating arms with paddles in appropriate locations in the container, etc. Modifying the flow pattern may improve transport of important decellularization agents in the solution and increase their transfer, improving percent decellurization. In addition, modifying the flow may enhance removal of cells and cellular components from the tissue.

In certain embodiments of the invention in which the construct is an engineered vessel grown in a bioreactor, the decellularization solution can be pumped through the inner lumen of the vessel to decellularize the inner portion of the vessel. In addition, the tissue culture medium can be removed from the bioreactor and replaced with decellularization solution to expose the outer portion of the vessel to decellularizing conditions. Application of pulsatile forces (described above) during the decellularization period may be employed to enhance decellularization.

Following decellularization and washing, the decellularized tissue engineered construct or decellularized engineered native tissue may be implanted into a subject in need thereof, e.g., as a replacement blood vessel, heart valve, organ, etc., or may be subjected to additional tissue engineering steps including seeding with cells. Alternatively, the decellularized construct can be stored for future use as described below.

Evaluating effects of decellularization

Various methods may be used to assess the effects of a particular decellularization protocol in regards to the extent of decellularization achieved and/or in regards to the alterations in the non-cellular structural components (e.g., the extracellular matrix). Samples of the tissue may be stained, e.g., with hematoxylin

and eosin, and examined by light microscopy. When hematoxylin and eosin staining is employed extracellular matrix components appear pink, and nuclei appear as purple spots as shown in Figures 2, 3, and 4. Staining procedures and stains that differentiate between cells and extracellular matrix, and stains that differentiate between various extracellular matrix components (e.g. collagen and elastin) are well known in the art. The number of cells present in the tissue can be determined by visual inspection at about 20X to 100X magnification. To assess the percent decellularization achieved, the number of cells present in a given area of decellularized tissue is compared with the number of cells present in an equivalent area of control tissue that has not been subjected to decellularization. The integrity of the non-cellular structural components can also be assessed by visual inspection. For example, deterioration in these components may be evidenced by fragmentation or separation between fibrils of extracellular matrix material.

Other techniques for assessing the extent of decellularization include immunohistochemistry and electron microscopy. Immunohistochemistry may be used to detect specific cellular components including components that may be particularly immunogenic such as histocompatibility antigens. Details regarding processing of tissues for light and electron microscopy and for immunohistochemistry may be found in the references cited at the beginning of this section, in particular Bader, et al. Other appropriate techniques are known to those of ordinary skill in the art. An estimate of the density of cells remaining after decellularization may also be obtained by determining the DNA content of the tissue, e.g., by measuring the fluorescence intensity of a dye such as Hoechst 33258 upon binding to DNA as described in pending application "Tissue-Engineered Constructs", Ser. No. 09/109,427.

In certain embodiments of the invention the removal of cells and cellular components results in reduced immunogenicity of the decellularized construct as compared with the construct before decellularization. A variety of approaches may be used to demonstrate the reduced immunogenicity of the decellularized construct. For example, the humoral immune response to extracts made from decellularized constructs may be compared with the humoral immune response to extracts made from control constructs that have not been decellularized (See Example 4 in U.S. Patent No. 5,613,982). Briefly, rabbits are immunized with NaCl extracts of either decellularized or control constructs, and immune sera are obtained. The immune sera

are screened for the presence of IgG and IgM antibodies against antigens present in extracts made from non-decellularized (control) constructs. Another approach to assessing the reduction in immune and inflammatory responses to decellularized constructs compared with control constructs involves implanting samples of the constructs into rabbits, removing the implants and surrounding tissue after a period of time such as two weeks, and subjecting the removed implants and tissue to histopathologic analysis (See Example 5 in U.S. Patent No. 5,613,982). The presence of inflammatory and immune system cells in the samples serves as an indicator of the degree of the inflammatory and immunologic response triggered by the implants. A variety of other methods known to those skilled in the art may be employed to assess the reduction in immunogenicity and inflammatory response due to decellularization of the tissue engineered constructs.

Storage of a decellularized construct

A decellularized construct or decellularized native tissue may be stored after decellularization using any of a number of storage techniques. Storage of decellularized constructs or tissues would provide ready access to these materials when needed. In a particularly advantageous embodiment of the invention, many tissue engineered constructs are prepared using human cells obtained from a single preferred source (e.g., a single human donor whose cells have been screened to reduce the likelihood of transmission of infectious diseases or a cell line that exhibits particularly preferred properties or has been genetically modified to enhance its ability to secrete extracellular matrix components). The tissue engineered constructs are decellularized and stored. When a subject who would benefit from implantation of a tissue engineered construct is identified, a stored construct is reconstituted and either implanted directly into the patient or subjected to further tissue engineering, e.g., seeding with cells obtained from the patient.

Cryopreservation (i.e., preserving by maintaining at an extremely low temperature) is a method for storing the decellularized construct or decellularized native tissue. Freezing and vitrification are two different approaches currently being pursued. In both cases, prevention of destructive ice crystal formation is a major goal. For freezing, the tissue or organ to be cryopreserved is perfused with a solution containing a sufficient concentration (generally approximately 10% by volume) of a

cryoprotective agent (CPA) so that ice formation is limited during subsequent cooling. Typical cryoprotectants include glycerol, dimethylsulfoxide (DMSO), glycols, propanediol, polyvinylpyrrolidone, dextran, and hydroxyethyl starch. Vitrification is a cryopreservation technique involving solidification in an amorphous glassy state that minimizes or eliminates ice crystal formation and growth. In both cases, tissues must be typically cooled to temperatures below -100°C (e.g., in liquid nitrogen) for long-term stability. For vitrification, the tissue is perfused with even higher concentrations of CPA than for freezing. Following incubation in the cryopreservation solution, the tissue may be packaged in a sterile container. In a preferred embodiment of the invention in which a tissue engineered construct is grown and decellularized in a bioreactor, the cryopreservation solution is introduced into the bioreactor, which is used as the storage container.

The choice and concentration of cryoprotectant, time-course for the addition of cryoprotectant, temperature at which the cryoprotectant is introduced, and rate of cooling and subsequent rewarming all play an important role in the success of preservation procedures. A variety of specific procedures and methods for preservation and reconstitution after storage have been developed and applied to various tissues and cells. Techniques for preserving tissues and organs, including blood vessels, heart valves, muskuloskeletal tissues, and collagenous tissues, by cryopreservation are described, for example, in U.S. Patent Nos. 4,890,4575; 5,131,850; 5,145,769; 5,158,867 and in U.S Patent No. 5,336,616, which discloses a method for preserving an acellular, collagen-based tissue matrix. The method includes incubating a decellularized tissue comprising a proteinaceous matrix with a cryoprotectant solution, followed by freezing at cooling rates such that minimal functional damage occurs to the proteinaceous matrix, drying the cryoprepared tissue under temperature and pressure conditions that permit removal of water without substantial ice recrystallization or ultrastructural damage, storage of the tissue, and subsequent rehydration.

Techniques and reagents for vitrification are described in U.S. Pat. No. 4,559,298; U.S. Pat. No. 5,217,860, U.S. Patent No. 5,952,168, and U.S. Patent No. 5,962,214. The contents of the afore-mentioned nine patents are herein incorporated by reference. The methods disclosed in these references will be readily adaptable to the decellularized, tissue engineered constructs and decellularized, engineered native

tissues disclosed herein.

Although cryopreservation represents a reliable approach to storing a decellularized tissue engineered construct of the present invention, alternative methods are also within the scope of the invention. For example, drying methods can also be used, with the addition of stabilizing compounds such as sucrose. A dextran and sucrose combination provides desirable physical properties and protein protection against freeze drying and air drying stresses. Freeze drying may take place using a lyophilizer. Air drying may take place under a stream of dry nitrogen, and the construct may then be lyophilized under a vacuum at room temperature.

Reconstitution of a decellularized construct

Depending upon the particular storage technique selected, the construct is appropriately reconstituted before being implanted into a subject or used for further tissue engineering. Reconstitution preferably removes cryopreservation agents that are potentially toxic to cells and irritating if introduced into the body. In addition, preferred reconstitution techniques cause minimal alteration in the structural components of the construct. In the case of cryopreservation, reconstitution includes warming (preferably rapidly) and removal of the cryopreservation solution as described in the patents listed above. Removal of the cryopreservation solution may be accomplished by thorough washing, e.g, in normal saline or standard cell culture medium. If drying is employed, reconstitution includes rehydration (e.g., in normal saline or standard cell culture medium), as described in U.S. Patent No. 5,336,616. Antibiotics and/or antifungal agents may be included in the solutions used for rinsing and/or rehydration to minimize the chance of contamination. Dried tissue can be exposed directly to a cell suspension, thereby reconstituting and seeding in one step. The dried tissue can be rinsed, e.g., with media to remove any drying agents and then soaked in media if needed before exposing the tissue to a cell suspension.

Uses and further engineering of a decellularized tissue engineered construct

As mentioned above, a decellularized tissue engineered construct can be implanted into the body of a subject in order to repair, replace, or augment a tissue or organ in need thereof. Implantation can be performed using any of a variety of techniques, e.g., surgical techniques, known to those of skill in the art and dependent

upon the particular function that the construct is intended to fulfill. For example, a decellularized vascular construct may be used in a bypass operation to replace a diseased blood vessel. A decellularized heart valve construct may be used in a valve replacement operation, e.g., to replace a stenotic or incompetent valve. In the case that the decellularized construct is implanted directly into a recipient, the construct may be repopulated *in vivo* with the recipient's own cells. Various agents such as growth factors, etc., may be applied to the construct to enhance this process. Such agents may be applied, for example, prior to implantation, or after implantation, e.g., by injection into or near the construct, by systemic delivery to the recipient of the construct, etc.

In certain embodiments of the invention the decellularized construct is subjected to further tissue engineering steps prior to implantation into a recipient. Such steps can comprise seeding the construct with one or more populations of cells, preferably cells obtained from the intended recipient. For example, a decellularized vascular construct can be seeded with smooth muscle and/or endothelial cells obtained from a biopsy specimen taken from the intended recipient. After a period of time during which the cells are allowed to adhere to the construct the seeded construct can be implanted into the recipient. In certain embodiments of the invention the cells are genetically transformed so that they exhibit desirable characteristics, e.g., production of a protein or other molecule that is lacking in the recipient or production of a growth factor that stimulates cellularization or angiogenesis in the construct. In other embodiments of the invention the construct is impregnated with a bioactive agent such as a pharmaceutical composition prior to implantation into the recipient and thereby serves as a drug delivery vehicle.

In certain embodiments of the invention the decellularized construct is seeded and cultured for a period of time prior to implantation into the recipient. This growth period may be relatively short (e.g., 1 - 2 weeks) compared with the time required to grow the construct prior to decellularization. This period allows the seeded cells to become established and commence division. However, since the construct already possesses substantial mass and strength, it is not necessary to culture the cells for long enough to generate an extensive extracellular matrix. Of course the decellularized construct can be subjected to multiple seedings and growth periods. In general, any or all of the techniques employed in the growth of the construct prior to decellularization

may be employed during any growth phases that follow decellularization. For example, substances described above may be applied to the decellularized construct to promote adherence of cells. Growth factors may be applied to the construct and/or included in the medium to promote the growth of cells and/or the development or maintenance of a differentiated phenotype. In certain embodiments of the invention stimuli such as those described above (e.g., pulsatile forces and/or fluid flow) are applied to the seeded, decellularized construct during the growth period(s) that follow decellularization.

Thus certain embodiments of the present invention involve producing a tissue engineered blood vessel using a bioreactor system in which pulsatile and fluid flow stimuli are applied to a substrate that is seeded with smooth muscle cells and endothelial cells that are allogeneic to an intended recipient. The substrate is cultured with the application of pulsatile stretch for approximately 6 - 8 weeks, during which a substantial proteinaceous extracellular matrix is secreted, and the construct attains desired physical properties and thickness. The construct is then decellularized in the bioreactor chamber, with the application of pulsatile forces and fluid flow during the decellularization period to enhance the decellularization process and contribute to removal of the substrate. The decellularized construct is stored until needed. Following identification of an individual in need of a vascular graft, the construct is retrieved from storage and is seeded with smooth muscle and endothelial cells obtained from the intended recipient. After a relatively short culture period (e.g., 1 - 2 weeks), during which pulsatile stimuli and/or fluid flow may be applied to the construct, the recellularized construct is implanted into the recipient using an appropriate surgical procedure.

Assessing biomechanical properties and cell viability of constructs or tissues

It may be desirable to employ a decellularized construct that displays biomechanical properties similar to or superior to those of the tissue or organ to which they correspond, particularly when the decellularized construct is to be implanted into the body without being subjected to additional tissue engineering steps, . A variety of methods may be employed to test the biomechanical properties of decellularized tissue engineered constructs or of decellularized constructs that have been subsequently cell seeded and cultured. The particular technique selected will, in

general, depend upon the construct, and the desired biomechanical properties will depend upon the intended function of the construct following implantation into a recipient. Suitable methods for testing the biomechanical properties of a vascular construct are described in pending application "Tissue-Engineered Constructs", Ser. No. 09/109,427 (see Examples therein) and include measurement of burst strengths and compliances and measurement of suture retention strength. Stress-strain analyses such as the single load versus elongation test, the stress relaxation test, and the tensile failure test are described in U.S. Patent No. 5,613,982 (see Example 7) are also appropriate and may be applied, in general, to any type of tissue engineered construct. Additional tests known to those of skill in the art may also be used.

In those embodiments of the invention in which the decellularized construct is seeded and cultured prior to implantation into a recipient, it my be desirable that the construct is functional and viable prior to implantation. Various methods may be used to assess the functioning and viability of the construct. For example, cell viability may be assessed by trypan blue exclusion assay, by measuring total protein synthesis (e.g., by measuring incorporation of [^{3}H] proline) or DNA synthesis (e.g., by measuring incorporation of [^{3}H] thymidine). More specific assays of cellular activity such as measurement of collagen production are also well known in the art as described in U.S. Patent No. 5,613,982.

<u>Production of a decellularized, engineered native tissue</u>

The inventive methods described above involve the decellularization of a tissue engineered construct. However, the methods may be extended to include the decellularization of a native tissue that has been harvested from a donor and subjected to tissue engineering steps prior to decellularization. Methods for harvesting tissues from donors (e.g., living or cadaveric animal or human donors) are well known in the art. Various methods have been employed to harvest vascular tissues, heart valves, skin, organs such as kidneys, livers, lungs, hearts, etc. In some cases tissues or organs are harvested for purposes of transplanting them directly into a recipient, in which case the goal is generally to preserve the tissue or organ in a state as closely approximating the state in which it was removed from the donor as possible, and the harvested tissue or organ is subjected to minimal processing. In other instances, e.g., the harvesting of porcine heart valves to be used as replacements for human heart

valves, the harvested tissue may be subjected to extensive chemical processing such as fixation, decellularization, cross-linking, etc., to reduce immunogenicity and/or to improve physical characteristics. Processes for decellularizing harvested native tissue and repopulating it with new cells have been described (e.g., in U.S. Patent No. 5,192,312 and U.S. Patent No. 5,613,982). However, treatment of harvested tissues prior to decellularization has generally been limited to storage and/or preservation of the tissue.

According to the present invention, harvested native tissue from an animal or human donor is grown in culture. In certain embodiments of the invention the native tissue is seeded with cells before or after a culture period, although this is not a requirement. The cells may be of any of the types described above, but preferably the cells are derived from the same species as the intended recipient of the engineered tissue. In general, any of the culture methods and techniques described in the context of producing a tissue engineered construct may be employed in culturing harvested native tissue. For example, growth factors may be employed to promote cell growth or maintenance of a differentiated phenotype. Agents selected to promote adherence of cells may be applied to the native tissue. The harvested native tissue may be subjected to physical stimuli such as pulsatile stretch or fluid flow during the culture period as described above for tissue engineered constructs. Following one or more culture periods, during which one or more cell seedings may be performed, the native tissue is decellularized. The decellularized, engineered native tissue may be used for any of the purposes described above for decellularized tissue engineered constructs.

Thus in summary this inventive method includes the steps of (i) harvesting a native tissue; (ii) subjecting the native tissue to one or more tissue engineering steps to produce an engineered native tissue; and (iii) decellularizing the engineered native tissue to produce a decellularized, engineered native tissue. The tissue engineering step(s) comprise culturing the native tissue under conditions suitable for growth and can optionally include subjecting the native tissue to one or more cell seedings with optional intervening growth periods, subjecting the tissue to mechanical, electrical, and/or chemical stimuli. Such stimuli can include the application of pulsatile stretch or fluid flow forces, treating the native tissue with growth factors, etc. The decellularized, engineered native tissue can be used in any of the ways described above for a decellularized tissue engineered construct. Thus the decellularized,

engineered native tissue can be implanted into the body of a subject or can be used for further tissue engineering. The decellularized, engineered native tissue can be stored and reconstituted as described above. The decellularized, engineered native tissue can be seeded with cells, e.g., cells derived from the intended recipient, and can be maintained in culture prior to implantation into the recipient. Mechanical, electrical, and/or chemical stimuli can be applied during the culture period(s) following decellularization.

EXAMPLES

Example 1

Preparation of a Primary Cell-Seeded Construct

This example describes the preparation of a tissue engineered construct suitable for decellularization, in this case a small caliber artery, using a bioreactor system. A more detailed description of many aspects of this process is found in the pending application referenced above. As an initial step, a non-woven mesh made of fine polyglycolic acid (PGA) fibers (Albany International Research Co., Mansfield, MA) was produced and further processed to yield a porous substrate with a hydrophilic surface. The processing enhances wettability and increases the number of cells which are deposited on the surface during seeding. Briefly, the treatment began with three successive 30 minute washes in hexane, dichloromethane, and diethyl ether followed by lyophilization overnight. The PGA mesh was then placed briefly in ethanol, removed to distilled water, and placed in a 1.0 normal solution of NaOH for 1 minute, during which the solution was agitated. The mesh was then washed successively in distilled water, changing the solution until the pH of the wash solution remained at approximately 7.0. The mesh was then lyophilized overnight. The mesh was rolled into tubes with inner diameters of approximately 3-6 mm and lengths of approximately 1-10 cm which were then sewn together with uncoated PGA suture (Davis & Geck, Inc., Manati, P.R.) to form a tubular substrate.

Figure 1 depicts the bioreactor system (10), assembled appropriately for cell seeding.

The bioreactor includes a glass chamber (22) with a volume of approximately 200 ml and hollow side-arms (12) with a 4 mm internal diameter. The side-arms are attached to tubular plastic connectors (24) within the vessel. A short tubular sleeve (26) of non-degradable Dacron vascular graft material (Sherwood-Davis & Geck, St. Louis, MO) having an approximately 5 mm internal diameter is sutured to the plastic connector on either side of the bioreactor. The Dacron sleeve, which is highly porous, functions as an anchor to attach the developing tissue to the plastic and glass of the bioreactor system. Smooth muscle cells and fibroblasts grow easily into the pores, thus allowing formation of a continuous connection between the cellular tissue and the non-degradable elements of the system.

In preparation for production of a vascular scaffold the ends of the tubular PGA substrate (16) were sutured to the Dacron sleeves using uncoated Dacron suture (Sherwood-Davis & Geck, St. Louis, MO). A length of highly distensible medical-grade silicone tubing (14) with a known compliance (Patter Products, Beaverton, MI) was inserted through the side-arms, plastic connectors, and Dacron sleeves. The bioreactor system, including the tubular substrate, was sterilized by exposure to ethylene oxide and allowed to outgas for at least 3 days.

Bovine aortic smooth muscle cells were obtained as follows. Explants of bovine thoracic aorta were obtained from a local abattoir on ice. Aortas were placed in PBS supplemented with penicillin at standard concentrations (100 U/ml). The intimal layer of the aortas was stripped away with forceps, and the outer adventitia was removed along with the outer media. The remaining middle portion of the media was laid down in a petri dish with the previously endothelial side down, and the tissue was scored at 1 cm intervals. Sufficient DMEM with Pen/Strep and 15% FBS was added to cover the bottom of the dish, without causing the tissues to float above the surface. The tissues were cultured for 7 to 10 days, during which smooth muscle cells migrated off the tissues to form a monolayer in the dish at the end of the culture period. The tissues were then removed, and the cells were cultured for a total of 2 - 3 passages. Smooth muscle identity and purity were confirmed by visual appearance and by immunostaining for smooth muscle α-actin. Cells were removed from culture by trypsinization (0.05% trypsin, 0.02% EDTA), centrifuged to a pellet, and gently resuspended to form a single cell suspension in fresh DMEM.

The substrate was seeded by evenly pipetting 1- 2 ml of a suspension of

bovine aortic smooth muscle cells at a concentration of approximately 5 X 10^6/ml onto the substrate. Cells were allowed to attach for approximately 30 minutes, and then fresh medium was added to the bioreactor vessel. The engineered vessel was cultured in the bioreactor for 8 weeks in an atmosphere of 10%CO_2 at a temperature of 37°C in DMEM supplemented with 20% FBS, penicillin G (100 U/ml), 5 mM HEPES, ascorbic acid (0.05 mg/ml), $CuSO_4$ (3 ng/ml), proline (0.05 mg/ml), alanine (0.03 mg/ml), and glycine (0.05 mg/ml) with continuous stirring. Ascorbic acid was replenished daily. Approximately half the medium was replaced with fresh medium twice per week. Thus a volume of fresh medium equivalent to the volume of the bioreactor was supplied each week.

Example 2

Decellularization of a Tissue Engineered Bovine Artery Construct Using Ionic Detergent Solutions

Materials and Methods

Small caliber arteries were engineered using bovine aortic smooth muscle cells as described in Example 1. After an 8 week culture period a vessel was removed from the bioreactor, washed with PBS, and sliced into segments 2 mm thick. The slices were immersed in 50 ml of a decellularization solution containing 1 M NaCl, 25 mM EDTA, 8 mM CHAPS in sterile PBS at pH 7.2. The samples were incubated with continuous stirring for 1 hour at room temperature and were then washed three times in PBS. The samples were then placed in 50 ml of a second decellularization solution containing 1 M NaCl, 25 mM EDTA, 1.8 mM SDS in sterile PBS at pH 7.2 and incubated for 1 hour at room temperature with continuous stirring. After removal from the decellularization solution the segments were washed twice with PBS for 5 minutes to remove residual solution.

The decellularized artery and a control artery that had been produced under identical conditions but not subjected to decellularization were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to standard techniques.

Results

Figure 2 shows low-power (66X, panel A) and high-power (100X, panel B) photomicrographs of untreated control vessels. In panel A, the outer surface of the vessel wall is at the right, and the inner (luminal) surface is to the left. As shown in the figure, the outermost portion of the vessel is composed almost entirely of cells (visible as small dark purple spots) and extracellular matrix. The innermost third of the vessel also contains circular polymer fragments, which are incorporated into the engineered vessel in a disorganized fashion. In the higher powered view of panel B, the outer surface of the vessel is oriented downward.

Figure 3 shows low-power (66X, panel C) and high-power (100X, panel D) photomicrographs of the decellularized vessel. The inner surface of the vessel is oriented towards the right in panel C. As shown in the figure, polymer fragments are more loosely incorporated into the vessel architecture than in the control artery. Panel D shows the tissue portion of the vessel with the outer surface on the right. Some cellular fragments are visible, especially in the outermost portions of the vessel wall, but the overall number of cells and cellular remnants is significantly reduced compared with the control artery.

Example 3

Decellularization of a Tissue Engineered Bovine Artery Construct Using a Nonionic Detergent Solution

Materials and Methods

Small caliber arteries were engineered using bovine aortic smooth muscle cells as described in Example 1. After an 8 week culture period a vessel was removed from the bioreactor, rinsed with PBS, and sliced into segments 2 mm thick. The slices were immersed in 50 ml of a decellularization solution containing 1% Triton X-100®(Sigma), 0.02% EDTA (Sigma), 20 μg/ml RNAse A (Sigma), and 0.2 mg/ml DNAse (Sigma) in sterile PBS without Ca^{2+} or Mg^{2+} and incubated for 24 hours in a 10% CO_2 atmosphere at 37°C with continuous stirring. After removal from the decellularization solution, the segments were washed several times with PBS to remove residual solution.

The decellularized artery and a control artery that had been produced under identical conditions but not subjected to decellularization were fixed in formalin,

embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to standard techniques.

Results

Figure 4 shows low-power (66X, panel E) and high-power (100X, panel F) photomicrographs of the decellularized vessel. The outer surface of the vessel is oriented towards the right in panel E. As shown in the figure, polymer fragments (34) are very loosely adherent to the remaining collagen matrix. Panel F shows the tissue portion of the vessel, with very few remnants of nuclear material remaining between collagen strands. In comparison with the control artery shown in Figure 2, the number of cells and cellular remnants is significantly reduced.

Example 4

Decellularization of a Tissue Engineered Porcine Artery Construct Using Ionic Detergent Solutions

Materials and Methods

Porcine carotid artery smooth muscle cells were obtained as follows. Explants of porcine carotid artery were obtained from a local abattoir on ice. Aortas were placed in PBS supplemented with penicillin at standard concentrations (100 U/ml). The intimal layer of the aortas was stripped away with forceps, and the outer adventitia was removed along with the outer media. The remaining middle portion of the media was laid down in a petri dish with the previously endothelial side down, and the tissue was scored at 1 cm intervals. Sufficient DMEM with Pen/Strep and 101% FBS was added to cover the bottom of the dish, without causing the tissues to float above the surface. The tissues were cultured for 7 to 10 days, during which smooth muscle cells migrated off the tissues to form a monolayer in the dish at the end of the culture period. The tissues were then removed, and the cells were cultured for a total of 2 - 3 passages. Smooth muscle identity and purity were confirmed by visual appearance and by immunostaining for smooth muscle α-actin. Cells were removed from culture by trypsinization (0.05% trypsin, 0.02% EDTA), centrifuged to a pellet, and gently resuspended to form a single cell suspension in fresh DMEM.

A small caliber artery was engineered using porcine carotid smooth muscle cells essentially as described in Example 1 except that the medium used was supplemented with 10% FBS rather than 20% FBS. After an 8 week culture period

the vessel was removed from the bioreactor, washed with PBS, and sliced into segments 2 mm thick. The slices were immersed in 50 ml of a decellularization solution containing 1 M NaCl, 25 mM EDTA, 8 mM CHAPS in sterile PBS at pH 7.2. The samples were incubated with continuous stirring for 4 hours at room temperature and were then washed three times in PBS. The samples were then placed in 50 ml of a second decellularization solution containing 1 M NaCl, 25 mM EDTA, 1.8 mM SDS in sterile PBS at pH 7.2 and incubated for 4 hours at room temperature with continuous stirring. After removal from the decellularization solution the segments were washed twice with PBS for 5 minutes to remove residual solution.

The decellularized artery was fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to standard techniques.

Results

Figure 5A shows a photomicrograph of a portion of the engineered vessel prior to decellularization. The image shows a donut-shaped cross-section of the vessel. The purple cell nuclei are clearly visible, and the extracellular matrix is stained pink. Figure 5B shows a photomicrograph of a portion of the same vessel after decellularization. The almost complete absence of nuclear material suggests that the decellularization protocol effectively removed the great majority of cells while leaving the extracellular matrix substantially intact. It is likely that shorter periods of immersion in the decellularization solutions (e.g., 30 min to 4 hours) would also yield acceptable results.

Using this protocol there is no evidence of remaining polymeric substrate after decellularization. This finding suggests that the decellularization periods employed with the porcine vessel may more effectively remove the substrate than the shorter time periods employed to decellularize the bovine vessel described above.

Example 5

Decellularization and Reseeding of a Cultured Native Tissue Construct

Materials and Methods

Porcine carotid artery smooth muscle cells were obtained as describe in Example 4. To facilitate visualization after seeding, cells were fluorescently labeled with red fluorescent dye PKH26-GL (Sigma) according to the manufacturer's

instructions.

A segment of native adult porcine carotid artery was decellularized in a two-phase treatment of solutions. The artery was harvested as follows, according to a protocol similar to that described in Swindle, M.M., Moody, D.C., Phillips, L.D., *Swine as Models in Biomedical Research*, Ames, Iowa, Iowa State University Press, 1992. Anesthesia was induced with Tiletamine 2.0 mg/kg + Zolazepam 8.8 mg/kg IM, supplemented with intermittent boluses of Xylazine 2.2 mg/kg IM every 1-2 hours as needed. Anesthesia was maintained with Forane, 2%, during the length of the procedures. The left lateral neck was prepped and draped. The left common carotid artery was exposed, ligated, and excised for a length of 2-3 cm. The incision was closed in layers.

The harvested artery was placed in DMEM for transport (~10 min) and then immediately placed into decellularization solution. For the first twenty-four hours, the artery was submerged in PBS-based 8mM CHAPS, 1M NaCl, and 25mM EDTA. The solution was then changed to 1.8mM SDS, 1M NaCl, and 25mM EDTA for an additional 24 hours. Both treatments were conducted under sterile conditions at 37°C and 10% CO_2 with stirring. After decellularization, the vessel was rinsed thoroughly in PBS.

A segment of vessel was suspended in a sterile bioreactor and seeded with fluorescently labeled porcine carotid artery smooth muscle cells by evenly pipetting 1- 2 ml of cell suspension at a concentration of approximately 7 X 10^6/ml onto the outer surface of the decellularized vessel. The cells were allowed to attach for twenty minutes, and the seeded vessel was placed in a bioreactor as described above. The bioreactor was filled with 250ml of DMEM culture medium supplemented with 10% FBS, penicillin G (100 U/ml), 5 mM HEPES, ascorbic acid (0.05 mg/ml), $CuSO_4$ (3 ng/ml), proline (0.05 mg/ml), alanine (0.03 mg/ml), and glycine (0.05 mg/ml). Ascorbic acid was replenished daily.

The vessel was cultured for three days in a 10%CO_2 atmosphere at a temperature of 37°C with stirring. The vessel was then removed from the bioreactor, and segments were frozen for histology by first submerging in OCT (optimum cutting temperature) compound a widely available formulation of water soluble glycols and resins and then placing in liquid nitrogen. The segments were sliced in a frozen state and mounted on slides, which were kept frozen until examined under the microscope.

Results

Figure 6 shows (A) a phase contrast view of a vessel cross section and (B) the corresponding fluorescent cross section. Since they were fluorescently labeled prior to seeding, seeded cells can be distinguished from residual cells remaining after the decellularization procedure. The presence of fluorescent cells on the vessel surface as seen in Figure 6B indicated that seeded cells attached to the decellularized native vessel, but no inward migration occurred.

Example 6

Preparation of a Decellularized Tissue Engineered Bovine Construct Using a Nonionic Detergent Solution

A small caliber artery is engineered using bovine aortic smooth muscle cells as described in Niklason, *et al.*, Functional arteries grown in vitro, *Science*, 284: 489-93,1999. Briefly, 1 - 2 ml of a suspension of smooth muscle cells (5 X 10^6 cells/ml) isolated from the medial layer of bovine aorta (as described in Ross, R., *et al.*, *J. Cell. Biol.* 50, 172, 1999, the contents of which are incorporated herein by reference) are pipetted onto a tubular polyglycolic acid substrate that is secured in a bioreactor chamber over a length of distensible silicone tubing. The surface of the substrate is modified with sodium hydroxide as described in Gao, J., *et al, J. Biomed Mater. Res.* 42, 417, 1998, the contents of which are herein incorporated by reference, to increase surface hydrophilicity. After an initial seeding period of 30 min, the bioreactor is filled with medium (DMEM modified as described in Example 1). The construct is cultured for 8 weeks during which pulsatile radial stress is applied to the developing construct at 165 beats per minute and 5% radial distention (strain) by pumping medium through the distensible silicone tubing in a pulsatile fashion. Following the 8 week culture period the silicone tubing is removed, and the flow of medium is applied directly through the cultured vessel. To produce an endothelial layer, a suspension of bovine aortic endothelial cells (3 X 10^6 cells/ml) is injected into the lumen, and the cells are allowed to adhere for 90 min. Luminal flow rate is gradually increased from 0.033 to 0.1 ml/sec over 3 days of culture, with corresponding shear stresses at the vessel wall of 1 X 10^{-2} N/m^2 to 3 X 10^{-2} N/m^2. The construct is cultured for an additional two weeks during which it is subjected to intraluminal flow.

Following the culture periods, the medium is drained from the bioreactor, and the construct is rinsed with sterile PBS. The bioreactor vessel is filled with a decellularization solution containing 1% Triton X-100® (Sigma), 0.02% EDTA (Sigma), 20 μg/ml RNAse A (Sigma), and 0.2 mg/ml DNAse (Sigma) in sterile PBS without Ca^{2+} or Mg^{2+}. Decellularization solution is also placed in a flow system attached to the bioreactor and is pumped through the inner lumen of the vessel at a flow rate of approximately 0.1 ml/sec. After 24 hours of exposure to the decellularization solution at 37°C in a 10% CO_2 atmosphere at 37°C with continuous stirring, the decellularization solution is removed from the bioreactor and flow system, and the system is rinsed with PBS. The application of intraluminal flow through the interior of the engineered vessel results in removal of substantially all of the remaining fragments of the polymeric substrate.

For cryopreservation, the decellularized construct is first immersed for 20 min in HEPES-buffered DMEM containing 1 M DMSO, 2.5% chondroitin sulfate, and 10% fetal bovine serum at 4°C and then cooled at a controlled rate of approximately 1.0°/min to -80°C and transferred to liquid nitrogen for storage. Thawing is accomplished by immersing the storage container in a waterbath at 37°C until all ice has disappeared, after which the container is transferred sequentially for 5 minute periods to DMEM containing 0.5 M, 0.25 M, and finally 0 M mannitol as an osmotic buffer. The decellularized vessel is implanted into the right saphenous artery of a Yucatan miniature pig as described in Niklason, *et al.*, Functional arteries grown in vitro,

Science, 284: 489-93,1999.

Example 7

Preparation and Subsequent Engineering of a Decellularized Tissue Engineered Construct

A vascular tissue engineered construct is produced in a plastic bioreactor system similar to that described in Example 1, but without the use of a polymer substrate, as follows: Human smooth muscle cells and human endothelial cells are obtained using standard biopsy and culture techniques and are maintained *in vitro*. The length of silicone tubing extending between the Dacron sleeves in the bioreactor

is coated in a sterile fashion with a thin layer of a gelatin material, e.g., made from dilute human collagen. Human collagen (commercially available) is denatured and pipetted or applied with a syringe onto the outside of the silicone tubing to create a layer approximately 50 μm thick. The tubing may be rotated during application of the collagen solution so that a layer of uniform thickness is produced. Thus according to this embodiment of the invention the collagen-coated tubing serves as the substrate.

After the collagen layer is allowed to dry, a suspension of 1 to 2 ml of culture medium containing human smooth muscle cells at a concentration of approximately 5 million cells/ml medium, is applied to the coated tubing and the Dacron sleeves on either end using a pipet. The tubing is preferably rotated during application of the cells to create an even distribution of cells. The thin coating allows an initial layer of cells to adhere to the outside of the tubing. The bioreactor stopper is replaced, and the cells are allowed to adhere for 30 - 60 minutes, after which the bioreactor and fluid reservoir are filled with culture medium (DMEM supplemented as described in Example 1). The bioreactor is placed in a tissue culture incubator and maintained at 37°C in a 10% CO_2 atmosphere for a period of about 1 to 7 days during which pulsatile radial stress is applied to the developing construct at 165 beats per minute and 5% radial distention (strain) by pumping medium through the distensible silicone tubing in a pulsatile fashion.

Following this initial growth period, the stopper is removed, and the medium is drained from the bioreactor. The surface of the developing tissue on the tubing is then reseeded with a suspension of human smooth muscle cells substantially equivalent to those used in the initial seeding, and these cells are allowed to adhere to the developing tissue. The bioreactor is then filled with medium, and the culture process is repeated with physical stimuli applied as in the first growth period. This sequence (i.e., reseeding followed by a growth period) is continued until a tissue of desired thickness (e.g., approximately 0.038 cm) is produced on the tubing (approximately 8 weeks).

After the vessel has reached the desired thickness the silicone tubing is removed, and the flow of medium is applied directly through the cultured vessel. To produce an endothelial layer, a human endothelial cell suspension of 3×10^6 cells/ml in DMEM is injected into the lumen of the construct, and the cells are allowed to adhere for 90 min. Luminal flow rate is gradually increased from 0.033 to 0.1 ml/sec

over 3 days of culture, with corresponding shear stresses at the vessel wall of 1 X 10^{-2} N/m^2 to 3 X 10^{-2} N/m^2. The construct is cultured for an additional two weeks during which it is subjected to intraluminal flow.

Following this second growth period, the medium is removed from the bioreactor chamber, and the construct is rinsed with PBS. The chamber is then filled with a decellularization solution containing 1 M NaCl, 25 mM EDTA, 1.8 mM SDS in sterile PBS at pH 7.2. Decellularization solution is also placed in a flow system attached to the bioreactor and is pumped through the inner lumen of the vessel at a flow rate of approximately 0.1 ml/sec in a pulsatile fashion. After 30 minutes of exposure to the decellularization solution at room temperature, the decellularization solution is removed from the bioreactor and flow system, and the system is rinsed with PBS.

For cryopreservation, the bioreactor chamber is first filled with HEPES-buffered DMEM containing 1 M DMSO, 2.5% chondroitin sulfate, and 10% fetal bovine serum at 4°C. Following a 20 minute period at 4°C, the chamber is cooled at a controlled rate of approximately 1.0°C/min to a temperature of -80°C and transferred to liquid nitrogen for storage. After identification of a subject in need of a vascular graft, the decellularized vessel is thawed by immersing the bioreactor chamber in a waterbath at 37°C until all ice has disappeared, after which the cryoprotection solution is drained from the chamber. For elution of the cryoprotection solution, the chamber is then filled sequentially for periods of 5 minutes with DMEM containing 0.5 M, 0.25 M, and finally 0 M mannitol as an osmotic buffer. The chamber is then filled with DMEM supplemented as described in Example 1.

Smooth muscle cells and endothelial cells are obtained from a biopsy specimen removed from the intended recipient of the construct. These cells are maintained in tissue culture and expanded. Following thawing of the decellularized construct, the outer surface of the construct is seeded with smooth muscle cells (1-2 ml of a suspension containing 5 X 10^6 cells/ml), which are allowed to adhere for 30 minutes. The bioreactor chamber is filled with culture medium, and the construct is maintained in culture for a period of 1 week. To produce an endothelial layer, a suspension of bovine aortic endothelial cells (3 X 10^6 cells/ml) is injected into the lumen, and the cells are allowed to adhere for 90 min. The construct is cultured for an additional three days with the application of pulsatile stimuli as during the growth

periods prior to decellularization and is then implanted into the recipient.

* * *

While the invention has been described and illustrated in connection with certain embodiments, many variations and modifications as will be evident to those skilled in this art may be made therein without departing from the spirit of the invention, and the invention as set forth in the claims is thus not to be limited to the precise details set forth above.

CLAIMS

We claim:

1. A method for producing a decellularized tissue engineered construct comprising the steps of:

providing a tissue engineered construct; and

decellularizing the tissue engineered construct, thereby forming a decellularized tissue engineered construct.

2. The method of claim 1, wherein the providing step comprises producing a tissue engineered construct, and wherein producing a tissue engineered construct comprises the steps of:

contacting a substrate with a population of cells capable of adhering thereto, thereby forming a cell-seeded construct; and

maintaining the cell-seeded construct under conditions suitable for growth of the population of cells for a growth period to form a tissue engineered construct.

3. The method of claim 1, wherein the providing step comprises producing a tissue engineered construct, and wherein producing a tissue engineered construct comprises the steps of:

contacting a substrate with a first population of cells capable of adhering thereto, thereby forming a primary cell-seeded construct; and

maintaining the cell-seeded construct under conditions suitable for growth of the first population of cells for a first growth period to form a primary tissue engineered construct;

contacting the primary tissue engineered construct with a second population of cells, thereby forming a secondary cell-seeded construct; and

maintaining the secondary cell-seeded construct under conditions suitable for growth of the second population of cells for a second growth period.

4. The method of claim 2, wherein the contacting and maintaining steps are repeated alternately until a cell-seeded construct having desired properties is formed.

5. The method of claim 4, wherein the contacting step is repeated using a plurality of different cell types.

6. The method of claim 2, wherein the substrate comprises a biocompatible material.

7. The method of claim 2, wherein the substrate comprises a porous material.

8. The method of claim 2, wherein the substrate comprises a collagen sponge.

9. The method of claim 2, wherein the substrate comprises a polymeric material.

10. The method of claim 2, wherein the substrate comprises a length of tubing.

11. The method of claim 10, wherein the length of tubing is coated.

12. The method of claim 2, wherein the substrate comprises a synthetic polymeric material.

13. The method of claim 12, wherein the synthetic polymeric material has a hydrophilic surface.

14. The method of claim 12, wherein the polymeric material comprises a polymer selected from the group consisting of polyesters of hydroxycarboxylic acids, polyanhyrides of dicarboxylic acids, and copolymers of hydroxy carboxylic acids and dicarboxylic acids.

15. The method of claim 2 wherein the substrate has an inner and outer surface, wherein the inner surface of the substrate defines a lumen.

16. The method of claim 2 wherein the substrate comprises a flat surface.

17. The method of claim 2 wherein the substrate comprises a three-dimensional structure.

18. The method of claim 2, wherein a mechanical force is applied to the construct during the growth period.

19. The method of claim 2, wherein a pulsatile stimulus is applied to the construct during the growth period.

20. The method of claim 2, wherein pulsatile stretch is applied to the construct during the growth period.

21. The method of claim 2, wherein the growth period is continued until the construct reaches a predetermined thickness.

22. The method of claim 2, wherein the growth conditions are chosen to promote deposition of extracellular matrix components.

23. The method of claim 2, wherein the cells are selected from the group consisting of: smooth muscle cells, cardiac muscle cells, epithelial cells, endothelial cells, urothelial cells, fibroblasts, myoblasts, chondrocytes, chondroblasts, osteoblasts, osteoclasts, hepatocytes, bile duct cells, pancreatic islet cells, thyroid, parathyroid, adrenal, hypothalamic, pituitary, ovarian, testicular, salivary gland cells, adipocytes, and precursor cells.

24. The method of claim 2, wherein the cells are neonatal cells.

25. The method of claim 2, wherein the population of cells comprises cells of at least two cell types.

26. The method of claim 2, wherein the cells are human cells.

27. The method of claim 2, wherein the cells are porcine cells.

28. The method of claim 2, wherein the cells are tumor cells.

29. The method of claim 2, wherein the cells are genetically transformed cells.

30. The method of claim 1 or 2, wherein the decellularization step comprises:

incubating the construct in a processing solution, the processing solution extracting cells from the construct.

31. The method of claim 30, wherein the processing solution comprises at least one component selected from the list consisting of: a detergent, a hypotonic solution, an RNA nuclease, and a DNA nuclease.

32. The method of claim 1 or 2, wherein at least 50% of the cells are removed in the decellularization step.

33. The method of claim 1 or 2, wherein at least 60% of the cells are removed in the decellularization step.

34. The method of claim 1 or 2, wherein at least 70% of the cells are removed in the decellularization step.

35. The method of claim 1 or 2, wherein at least 80% of the cells are removed in the decellularization step.

36. The method of claim 1 or 2, wherein at least 90% of the cells are removed in the decellularization step.

37. The method of claim 1 or 2, wherein at least 95% of the cells are removed in the decellularization step.

38. The method of claim 1 or 2, wherein at least 99% of the cells are removed in the decellularization step.

39. The method of claim 1 or 2, wherein substantially all of the cells are removed in the decellularization step.

40. The method of claim 2, further comprising the step of:

removing a portion of the substrate.

41. The method of claim 2, further comprising the step of:

removing substantially all of the substrate.

42. The method of claim 2, further comprising the step of:

applying a fluid shear to the decellularized construct, thereby removing a portion of the substrate.

43. The method of claim 2, further comprising the step of:

applying a fluid shear to the decellularized construct, thereby removing substantially all of the substrate.

44. The method of claim 2, further comprising the step of:

storing the decellularized tissue engineered construct.

45. The method of claim 44, further comprising the step of:

before storing the decellularized construct, pretreating the decellularized construct with an agent selected to protect the decellularized construct during the storage process.

46. The method of claim 44, wherein the storing comprises cryopreservation.

47. The method of claim 46, wherein the decellularized construct comprises a proteinaceous matrix, and wherein the storing step comprises:

incubating the construct in a cryoprotective solution and freezing at cooling rates such that minimal functional damage occurs to the proteinaceous matrix of the construct to produce a cryoprepared construct;

drying the cryoprepared construct under temperature and pressure conditions that permit removal of water without substantial ice recrystallization or ultrastructural damage.

48. The method of claim 44, wherein the storing comprises drying.

49. The method of claim 44, further comprising the step of:

reconstituting the decellularized construct after storage.

50. The method of claim 49, wherein the reconstituting step comprises:

incubating the decellularized construct in a rehydration solution, the rehydration solution reducing osmotic, hypoxic, autolytic, or proteolytic damage.

51. The method of claim 49, wherein the reconstituting step comprises:

incubating the decellularized construct in a rehydration solution, the rehydration solution reducing microbial contamination.

52. The method of claim 44, further comprising the step of:

treating the decellularized construct with a biologically active agent.

53. The method of claim 52, wherein the biologically active agent is selected to stimulate recellularization of the construct.

54. The method of claim 52, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

55. The method of claim 1 or 2, further comprising the step of:

subjecting the decellularized construct to further tissue engineering.

56. The method of claim 1, wherein providing a tissue engineered construct comprises:

purchasing a tissue engineered construct.

57. The method of claim 1, wherein providing a tissue engineered construct comprises providing a tissue engineered construct that has been produced primarily by growth *in vitro*.

58. The method of claim 1, wherein providing a tissue engineered construct comprises providing a tissue engineered construct that has been produced at least in part by growth *in vivo*.

59. A method for treating a subject suffering from tissue damage or loss comprising:

producing a decellularized construct according to the method of claim 1 or 2; and

implanting the decellularized construct into a subject in need thereof.

60. The method of claim 59, wherein the implanting step comprises supplementing or replacing a blood vessel of the subject.

61. The method of claim 59, wherein the implanting step comprises supplementing or replacing a tissue of the subject, the tissue selected from the list consisting of: a heart valve, a muscle, a joint, a ligament, a tendon, a bone, and an organ.

62. A method for producing an engineered construct comprising the steps of:

producing a tissue engineered construct;

decellularizing the tissue engineered construct, thereby forming a decellularized construct;

contacting the decellularized construct with cells capable of adhering thereto, thereby forming a cell-seeded decellularized construct; and

maintaining the cell-seeded decellularized construct for a growth period in an environment suitable for growth of the cells to form an engineered construct.

63. The method of claim 62, wherein the producing step comprises:

contacting a substrate with a population of cells capable of adhering thereto, thereby forming a cell-seeded construct; and

maintaining the cell-seeded construct under conditions suitable for growth of the population of cells for a growth period to form a tissue engineered construct.

64. The method of claim 62, wherein the producing step comprises:

contacting a substrate with a first population of cells capable of adhering thereto, thereby forming a primary cell-seeded construct; and

maintaining the cell-seeded construct under conditions suitable for growth of the first population of cells for a first growth period to form a primary tissue engineered construct;

contacting the primary tissue engineered construct with a second population of cells, thereby forming a secondary cell-seeded construct; and

maintaining the secondary cell-seeded construct under conditions suitable for growth of the second population of cells for a second growth period.

65. The method of claim 62, wherein the cells comprise human cells.

66. The method of claim 62, wherein the cells comprise genetically transformed cells.

67. The method of claim 62, wherein the cells are obtained by harvesting cells from a subject, the subject being the intended recipient of the tissue engineered construct.

68. The method of claim 62, wherein the cells are selected from the group consisting of: smooth muscle cells, cardiac muscle cells, epithelial cells, endothelial cells, urothelial cells, fibroblasts, myoblasts, chondrocytes, chondroblasts, osteoblasts, osteoclasts, hepatocytes, bile duct cells, pancreatic islet cells, thyroid, parathyroid, adrenal, hypothalamic, pituitary, ovarian, testicular, salivary gland cells, adipocytes, and precursor cells.

69. The method of claim 68, wherein the cells comprise cells of at least two different cell types.

70. The method of claim 63 or claim 64, further comprising the step of:

removing a portion of the substrate.

71. The method of claim 63 or claim 64, further comprising the step of:

removing substantially all of the substrate.

72. The method of claim 63 or claim 64, further comprising the step of:

applying a fluid shear to the decellularized construct, thereby removing a portion of the substrate.

73. The method of claim 63 or claim 64, further comprising the step of:

applying a fluid shear to the decellularized construct, thereby removing substantially all of the substrate.

74. The method of claim 62, further comprising the step of:

after decellularizing the tissue engineered construct to obtain a decellularized construct, storing the decellularized construct under conditions selected to preserve the construct.

75. The method of claim 74, further comprising the step of:

before storing the decellularized construct, pretreating the decellularized construct with an agent selected to protect the construct during the storage process.

76. The method of claim 74, wherein the storing comprises cryopreservation.

77. The method of claim 74, wherein the storing comprises drying.

78. The method of claim 74, further comprising the step of:

reconstituting the decellularized construct after storage.

79. The method of claim 78, further comprising the step of:

treating the decellularized construct with a biologically active agent.

80. The method of claim 79, wherein the biologically active agent is selected to stimulate recellularization of the construct.

81. The method of claim 79, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

82. A method for producing a decellularized engineered native tissue comprising the steps of:

procuring a tissue harvested from an animal or human;

engineering the harvested tissue, thereby forming an engineered native tissue; and

decellularizing the engineered native tissue, thereby forming a decellularized engineered native tissue.

83. The method of claim 82, wherein the engineering step comprises:

seeding the harvested native tissue with cells; and

maintaining the tissue under conditions suitable for growth of the cells for a growth period.

84. The method of claim 82, wherein the engineering step comprises:

subjecting the harvested tissue to a mechanical force, the mechanical force selected to enhance the properties of the tissue.

85. The method of claim 82, wherein the engineering step comprises:

subjecting the harvested tissue to an electrical stimulus.

86. The method of claim 82, wherein the engineering step comprises:

subjecting the harvested tissue to a pulsatile stimulus.

87. The method of claim 82, wherein the engineering step comprises:

treating the harvested tissue with a biologically active agent.

88. The method of claim 87, wherein the biologically active agent is selected from the list consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

89. The method of claim 87, wherein the biologically active agent comprises:

a pharmaceutical composition.

90. The method of claim 82, wherein the harvested tissue comprises a blood vessel.

91. The method of claim 82, wherein the harvested tissue comprises a heart valve.

92. The method of claim 82, wherein the harvested tissue comprises an organ or a portion thereof.

93. The method of claim 82, wherein the harvested tissue comprises a muscle.

94. The method of claim 82, further comprising the step of:

subjecting the decellularized engineered native tissue to further tissue engineering.

95. The method of claim 82, further comprising the step of:

seeding the decellularized engineered native tissue with cells.

96. The method of claim 95 wherein the cells are selected from the group consisting of: smooth muscle cells, cardiac muscle cells, epithelial cells, endothelial cells, urothelial cells, fibroblasts, myoblasts, chondrocytes, chondroblasts, osteoblasts, osteoclasts, hepatocytes, bile duct cells, pancreatic islet cells, thyroid, parathyroid, adrenal, hypothalamic, pituitary, ovarian, testicular, salivary gland cells, adipocytes, and precursor cells.

97. The method of claim 95, wherein the cells comprise cells of at least two different cell types.

98. The method of claim 95, wherein the cells comprise neonatal cells.

99. The method of claim 95, wherein the cells comprise human cells.

100. The method of claim 95, wherein the cells comprise genetically transformed cells.

101. A method for treating a subject suffering from tissue damage or loss comprising the steps of:

producing an engineered, decellularized construct according to the method of claim 62; and

implanting the tissue engineered construct into a subject in need thereof.

102. The method of claim 101, wherein the cells used in the final contacting step are obtained by harvesting cells from the subject.

103. The method of claim 101, wherein the cells used in the final contacting step are obtained by a method comprising the steps of:

harvesting cells from the subject; and

culturing the cells *in vitro* prior to seeding the decellularized construct.

104. The method of claim 101, wherein the implanting step comprises supplementing or replacing a blood vessel of the subject.

105. The method of claim 101, wherein the implanting step comprises supplementing or replacing a tissue of the subject, the tissue selected from the list consisting of: a heart valve, a muscle, a joint, a ligament, a tendon, a bone, and an organ.

106. The method of claim 101, further comprising the step of:

treating the engineered, decellularized construct with a biologically active agent before the implanting step, whereby the construct serves as a vehicle for delivery of the biologically active agent to the subject.

107. The method of claim 106, further comprising the step of:

treating the engineered, decellularized construct with a biologically active agent before the implanting step, wherein the biologically active agent is selected to enhance recellularization or vascularization of the construct after the implanting step.

108. The method of claim 106, wherein the biologically active agent comprises a pharmaceutical composition.

109. The method of claim 106, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

110. An engineered tissue for use as a tissue engineering scaffold or for implanting into a subject comprising:

a decellularized engineered native tissue.

111. The engineered tissue of claim 110, wherein at least 50% of the cells are removed from the decellularized engineered native tissue by decellularization.

112. The engineered tissue of claim 110, wherein at least 60% of the cells are removed from the decellularized engineered native tissue by decellularization.

113. The engineered tissue of claim 110, wherein at least 70% of the cells are removed from the decellularized engineered native tissue by decellularization.

114. The engineered tissue of claim 110, wherein at least 80% of the cells are removed from the decellularized engineered native tissue by decellularization.

115. The engineered tissue of claim 110, wherein at least 90% of the cells are removed from the decellularized engineered native tissue by decellularization.

116. The engineered tissue of claim 110, wherein at least 95% of the cells are removed from the decellularized engineered native tissue by decellularization.

117. The engineered tissue of claim 110, wherein at least 99% of the cells are removed from the decellularized engineered native tissue by decellularization.

118. The engineered tissue of claim 110, further comprising a biologically active agent.

119. The engineered tissue of claim 110, wherein the biologically active agent is selected to enhance recellularization or vascularization of the tissue engineered construct.

120. The engineered tissue of claim 110, wherein the biologically active agent comprises a pharmaceutical composition.

121. The engineered tissue of claim 110, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

122. The engineered tissue of claim 110, wherein the engineered native tissue comprises native tissue that has been subjected to a mechanical force after removal from an animal or human source, wherein the mechanical force is selected to enhance the properties of the tissue.

123. The engineered tissue of claim 110, wherein the engineered native tissue comprises native tissue that has been subjected to electrical stimulation after removal from an animal or human source.

124. The engineered tissue of claim 110, wherein the engineered native tissue comprises native tissue that has been treated with a growth factor after removal from an animal or human source.

125. The engineered tissue of claim 110, wherein the engineered native tissue comprises native tissue that has been exposed to serum after removal from an animal or human source.

126. The engineered tissue of claim 110, wherein the engineered native tissue comprises native tissue that has been exposed to a pulsatile stimulus after removal from an animal or human source.

127. The engineered tissue of claim 110, further comprising:

a population of cells, wherein the decellularized engineered native tissue is seeded with the population of cells after decellularization.

128. The engineered tissue of claim 127, wherein the decellularized engineered native tissue is maintained under conditions suitable for growth of the cells for a growth period following seeding.

129. The engineered tissue of claim 127, wherein the cells comprise human cells.

130. The engineered tissue of claim 127, wherein the cells comprise porcine cells.

131. The engineered tissue of claim 127, wherein the cells comprise neonatal cells.

132. A construct for use as a tissue engineering scaffold or for implanting into a subject comprising:

a tissue engineered construct that has been subjected to decellularization.

133. The construct of claim 132, wherein the tissue engineered construct comprises a substrate seeded with cells and maintained under conditions suitable for growth of the cells for a growth period.

134. The construct of claim 133, wherein the growth period comprises a period of time sufficient for formation of a tissue engineered construct having a predetermined thickness.

135. The construct of claim 133, wherein at least 50% of the cells are removed from the tissue engineered construct by decellularization.

136. The construct of claim 133, wherein at least 60% of the cells are removed from the tissue engineered construct by decellularization.

137. The construct of claim 133, wherein at least 70% of the cells are removed from the tissue engineered construct by decellularization.

138. The construct of claim 133, wherein at least 80% of the cells are removed from the tissue engineered construct by decellularization.

139. The construct of claim 133, wherein at least 90% of the cells are removed from the tissue engineered construct by decellularization.

140. The construct of claim 133, wherein at least 95% of the cells are removed from the tissue engineered construct by decellularization.

141. The construct of claim 133, wherein at least 99% of the cells are removed from the tissue engineered construct by decellularization.

142. The construct of claim 132, further comprising a biologically active agent.

143. The construct of claim 132, wherein the biologically active agent is selected to enhance recellularization or vascularization of the tissue engineered construct.

144. The construct of claim 132, wherein the biologically active agent comprises a pharmaceutical composition.

145. The construct of claim 132, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

146. The construct of claim 132, wherein the tissue engineered construct comprises a tissue engineered construct that has been subjected to a mechanical force during a growth period.

147. The construct of claim 132, wherein the tissue engineered construct comprises a tissue engineered construct that has been subjected to a pulsatile stimulus during a first growth period.

148. The construct of claim 132, wherein the tissue engineered construct comprises a tissue engineered construct that has been subjected to electrical stimulation during a first growth period.

149. The construct of claim 132, wherein the tissue engineered construct comprises a tissue engineered construct that has been treated with a growth factor during a first growth period.

150. The construct of claim 132, wherein the tissue engineered construct comprises a tissue engineered construct that has been exposed to serum during a first growth period.

151. The construct of claim 133, wherein the substrate comprises a polymeric material.

152. The construct of claim 133, wherein the substrate comprises a length of tubing.

153. The construct of claim 133, wherein the length of tubing is coated.

154. The construct of claim 133, wherein the substrate comprises a synthetic polymeric material.

155. The construct of claim 133, wherein the polymeric material comprises a polymer selected from the group consisting of polyesters of hydroxycarboxylic acids,

polyanhyrides of dicarboxylic acids, and copolymers of hydroxy carboxylic acids and dicarboxylic acids.

156. The construct of claim 133, wherein the substrate comprises a collagen sponge.

157. The construct of claim 133, wherein the substrate has an inner and outer surface, and wherein the inner surface of the substrate defines a lumen.

158. The construct of claim 133, wherein the substrate comprises a flat surface.

159. The construct of claim 133, wherein the substrate comprises a three-dimensional structure.

160. The construct of claim 133, wherein the construct is treated so as to remove substantially all of the substrate.

161. The construct of claim 133, wherein the cells are selected from the group consisting of: smooth muscle cells, cardiac muscle cells, epithelial cells, endothelial cells, urothelial cells, fibroblasts, myoblasts, chondrocytes, chondroblasts, osteoblasts, osteoclasts, hepatocytes, bile duct cells, pancreatic islet cells, thyroid, parathyroid, adrenal, hypothalamic, pituitary, ovarian, testicular, salivary gland cells, adipocytes, and precursor cells.

162. The construct of claim 133, wherein the cells comprise cells of at least two different cell types.

163. The construct of claim 133, wherein the cells comprise neonatal cells.

164. The construct of claim 133, wherein the cells comprise human cells.

165. The construct of claim 133, wherein the cells comprise porcine cells.

166. The construct of claim 133, wherein the cells comprise tumor cells.

167. The construct of claim 133, wherein the cells comprise genetically transformed cells.

168. A method for treating a subject suffering from tissue damage or loss comprising:

implanting the construct of claim 132 into a subject in need thereof.

169. The method of claim 168, further comprising the step of:

treating the construct with a biologically active agent before the implanting step, whereby the construct serves as a vehicle for delivery of the biologically active agent to the subject.

170. The method of claim 168, further comprising the step of:

treating the construct with a biologically active agent before the implanting step, whereby the biologically active agent is selected to enhance recellularization or vascularization of the construct after the implanting step.

171. The method of claim 168, wherein the biologically active agent comprises a pharmaceutical composition.

172. The method of claim 168, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

173. The method of claim 168, wherein the implanting step comprises supplementing or replacing a blood vessel of the subject.

174. The method of claim 168, wherein the implanting step comprises supplementing or replacing a tissue of the subject, the tissue selected from the list consisting of: a heart valve, a muscle, a joint, a ligament, a tendon, a bone, and an organ.

175. A method for treating a subject suffering from tissue damage or loss comprising:

implanting the engineered tissue of claim 110 into a subject in need thereof.

176. The method of claim 175, further comprising the step of:

treating the engineered tissue with a biologically active agent before the implanting step, whereby the engineered tissue serves as a vehicle for delivery of the biologically active agent to the subject.

177. The method of claim 175, further comprising the step of:

treating the engineered tissue with a biologically active agent before the implanting step, whereby the biologically active agent is selected to enhance recellularization or vascularization of the engineered tissue after the implanting step.

178. The method of claim 175, wherein the biologically active agent comprises a pharmaceutical composition.

179. The method of claim 175, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

180. The method of claim 175, wherein the implanting step comprises supplementing or replacing a blood vessel of the subject.

181. The method of claim 175, wherein the implanting step comprises supplementing or replacing a tissue of the subject, the tissue selected from the list consisting of: a heart valve, a muscle, a joint, a ligament, a tendon, a bone, and an organ.

182. A construct for use in tissue engineering or for implanting into a subject comprising:

a decellularized tissue engineered construct; and

a population of cells, wherein the decellularized tissue engineered construct is seeded with the population of cells.

183. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been subjected to a mechanical force during a growth period.

184. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been subjected to a pulsatile stimulus during a growth period.

185. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been subjected to electrical stimulation during a growth period.

186. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been treated with a growth factor during a growth period.

187. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been exposed to serum during a growth period.

188. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct produced using human cells.

189. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct produced using neonatal cells.

190. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct produced using genetically transformed cells.

191. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct produced using human cells.

192. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct produced using cells selected from the group consisting of: smooth muscle cells, cardiac muscle cells, epithelial cells, endothelial cells, urothelial cells, fibroblasts, myoblasts, chondrocytes, chondroblasts, osteoblasts, osteoclasts, hepatocytes, bile duct cells, pancreatic islet cells, thyroid, parathyroid, adrenal, hypothalamic, pituitary, ovarian, testicular, salivary gland cells, adipocytes, and precursor cells.

193. The tissue engineered construct of claim 182, wherein the cells comprise cells harvested from an intended recipient of the construct.

194. The construct of claim 182, wherein the population of cells is cultured *in vitro* before the decellularized tissue engineered construct is seeded therewith.

195. The construct of claim 182, wherein the population of cells is selected from the group consisting of: smooth muscle cells, cardiac muscle cells, epithelial cells, endothelial cells, urothelial cells, fibroblasts, myoblasts, chondrocytes, chondroblasts, osteoblasts, osteoclasts, hepatocytes, bile duct cells, pancreatic islet cells, thyroid, parathyroid, adrenal, hypothalamic, pituitary, ovarian, testicular, salivary gland cells, adipocytes, and precursor cells.

196. The construct of claim 182, wherein the population of cells comprises cells of at least two different cell types.

197. The construct of claim 182, wherein the population of cells comprises neonatal cells.

198. The construct of claim 182, wherein the population of cells comprises human cells.

199. The construct of claim 182, wherein the decellularized tissue engineered construct is maintained for growth period under growth conditions suitable for the growth of the population of cells.

200. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been subjected to a mechanical force during a growth period.

201. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been subjected to a pulsatile stimulus during a growth period.

202. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized construct that has been subjected to electrical stimulation during a growth period.

203. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been treated with a growth factor during a growth period.

204. The construct of claim 182, wherein the decellularized tissue engineered construct comprises a decellularized tissue engineered construct that has been exposed to serum during a growth period.

205. A method for treating a subject suffering from tissue damage or loss comprising:

implanting the construct of claim 182 into a subject in need thereof.

206. The method of claim 205, further comprising the step of:

treating the construct with a biologically active agent before the implanting step, whereby the construct serves as a vehicle for delivery of the biologically active agent to the subject.

207. The method of claim 205, further comprising the step of:

treating the construct with a biologically active agent before the implanting step, whereby the biologically active agent is selected to enhance recellularization or vascularization of the construct after the implanting step.

208. The method of claim 205, wherein the biologically active agent comprises a pharmaceutical composition.

209. The method of claim 205, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

210. The method of claim 205, wherein the implanting step comprises supplementing or replacing a blood vessel of the subject.

211. The method of claim 205, wherein the implanting step comprises supplementing or replacing a tissue of the subject, the tissue selected from the list consisting of: a heart valve, a muscle, a joint, a ligament, a tendon, a bone, and an organ.

212. A method for treating a subject suffering from tissue damage or loss comprising:

implanting the construct of claim 199 into a subject in need thereof.

213. The method of claim 212, further comprising the step of:

treating the construct with a biologically active agent before the implanting step, whereby the construct serves as a vehicle for delivery of the biologically active agent to the subject.

214. The method of claim 212, further comprising the step of:

treating the construct with a biologically active agent before the implanting step, whereby the biologically active agent is selected to enhance recellularization or vascularization of the construct after the implanting step.

215. The method of claim 212, wherein the biologically active agent comprises a pharmaceutical composition.

216. The method of claim 212, wherein the biologically active agent is selected from the group consisting of: growth factors, adhesion factors, soluble extracellular matrix proteins, thrombomodulators, antibiotics, and agents that augment hemocompatibility.

217. The method of claim 212, wherein the implanting step comprises supplementing or replacing a blood vessel of the subject.

218. The method of claim 212, wherein the implanting step comprises supplementing or replacing a tissue of the subject, the tissue selected from the list consisting of: a heart valve, a muscle, a joint, a ligament, a tendon, a bone, and an organ.

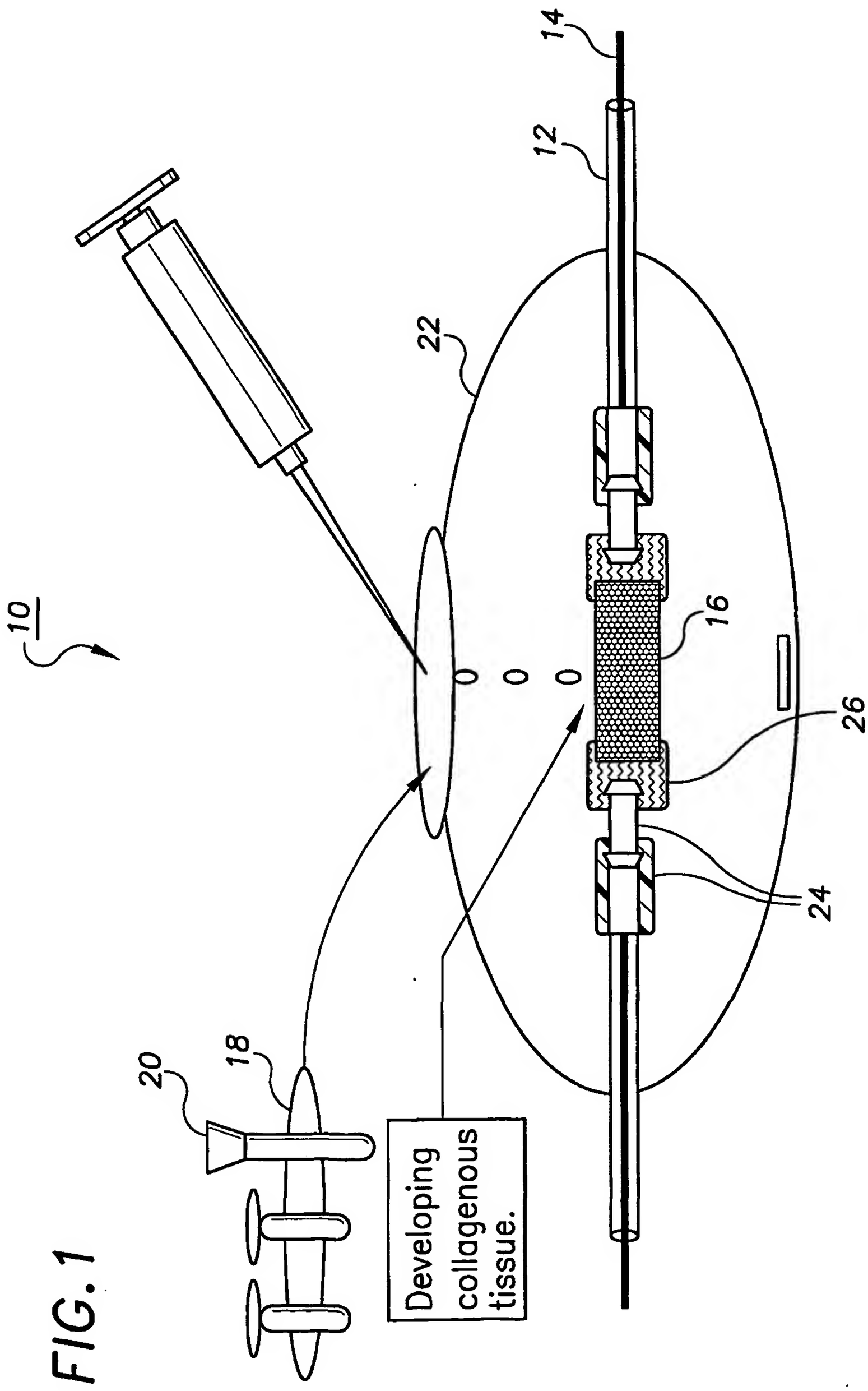

FIG. 1

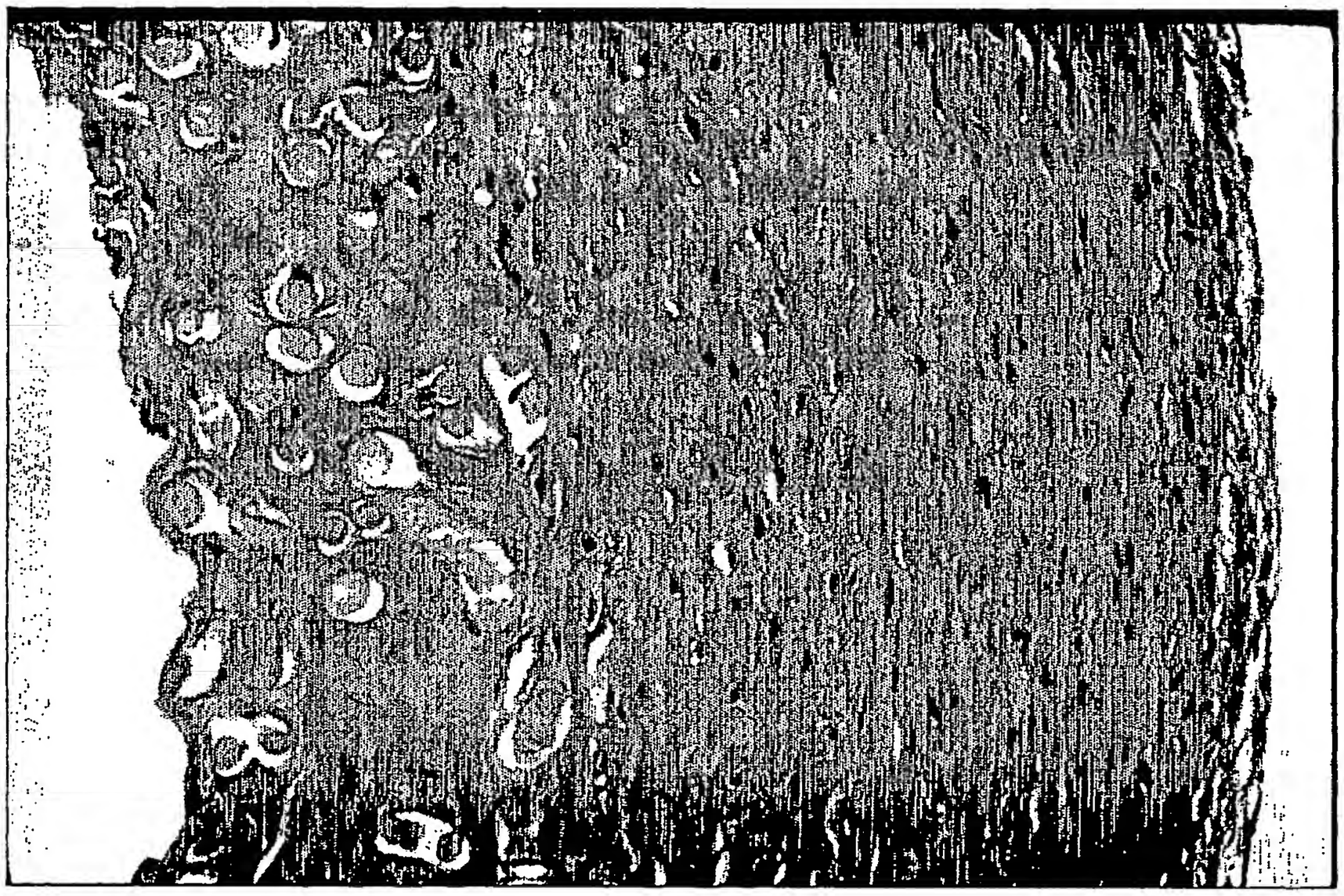

FIGURE 2A

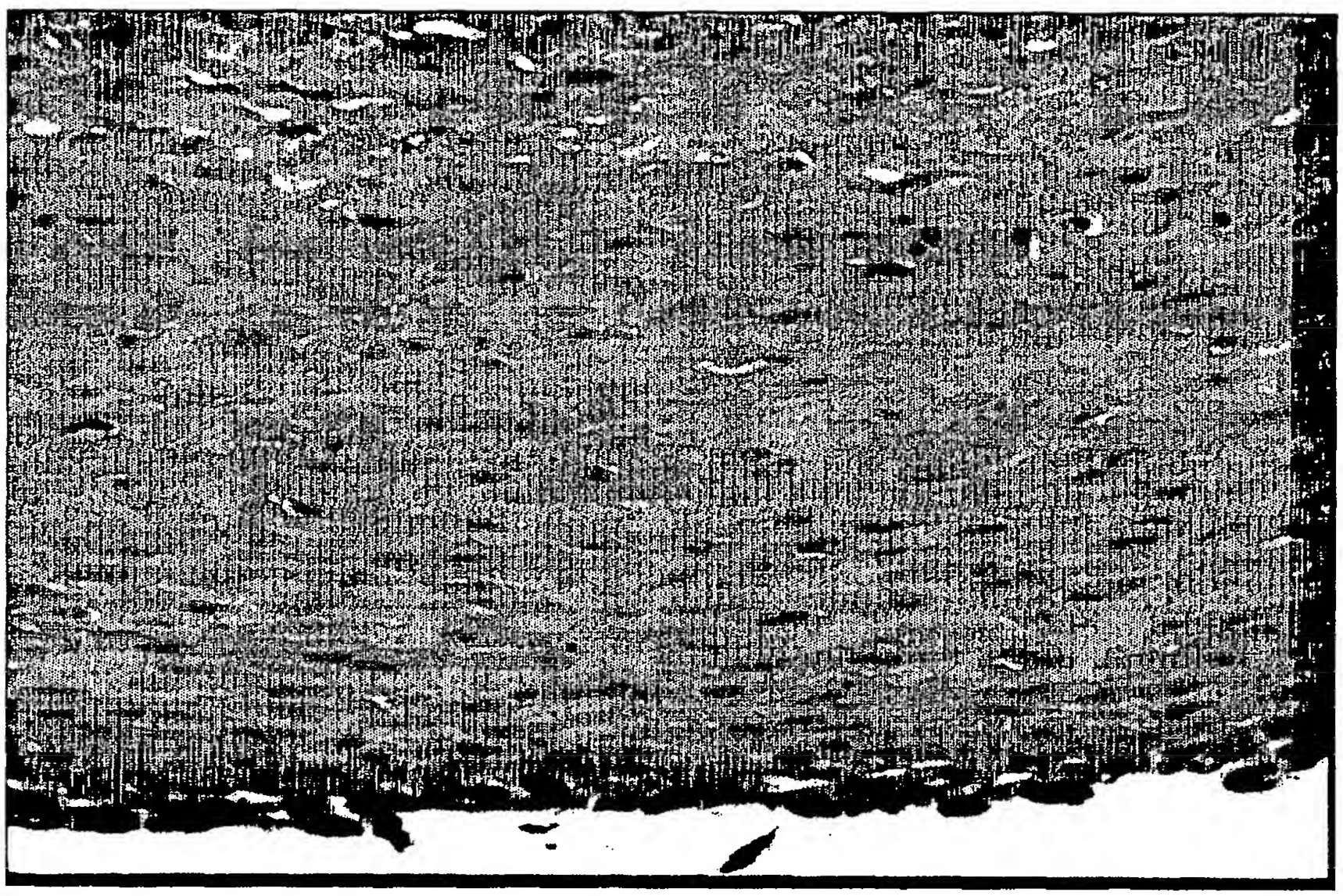

FIGURE 2B

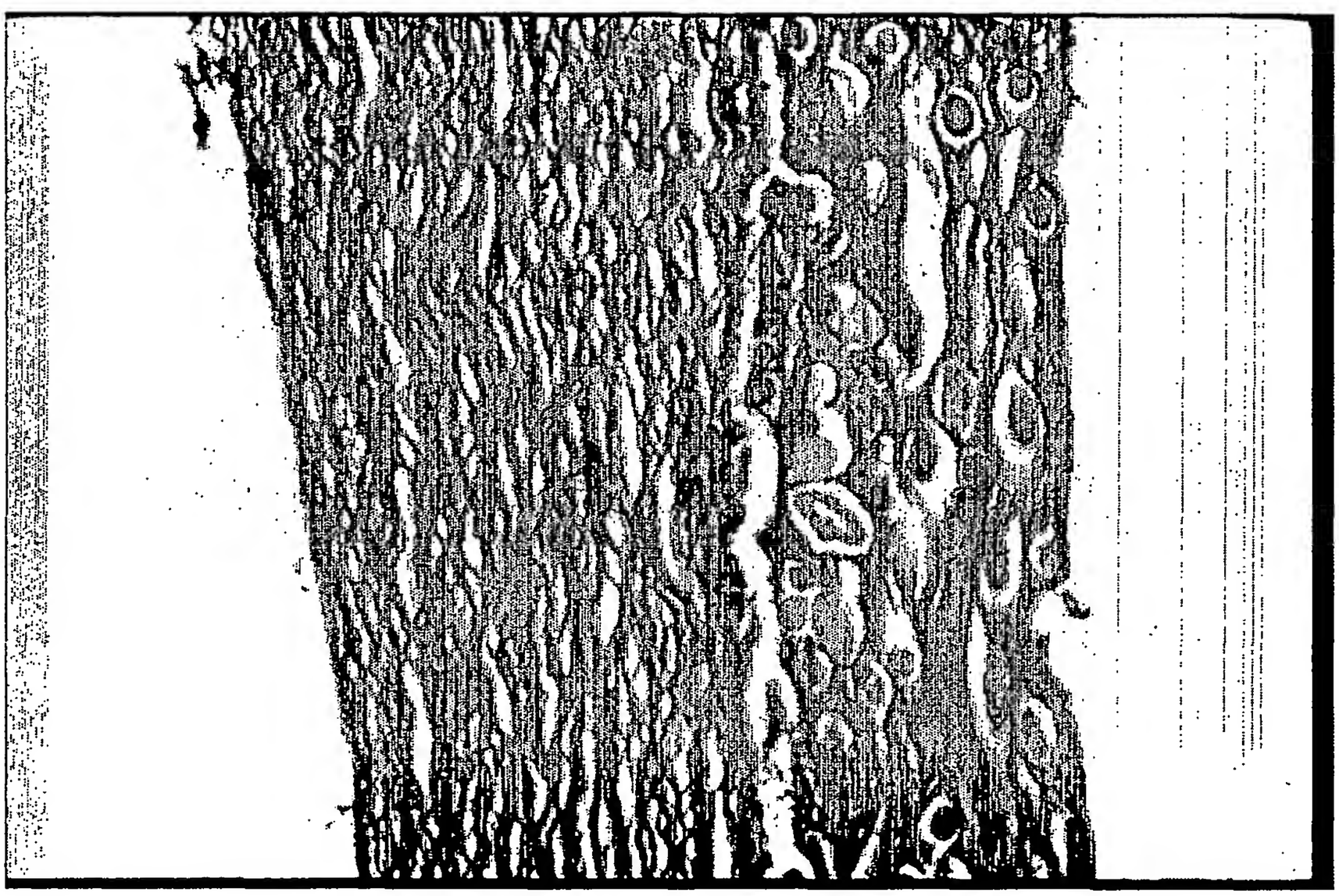

FIGURE 3A

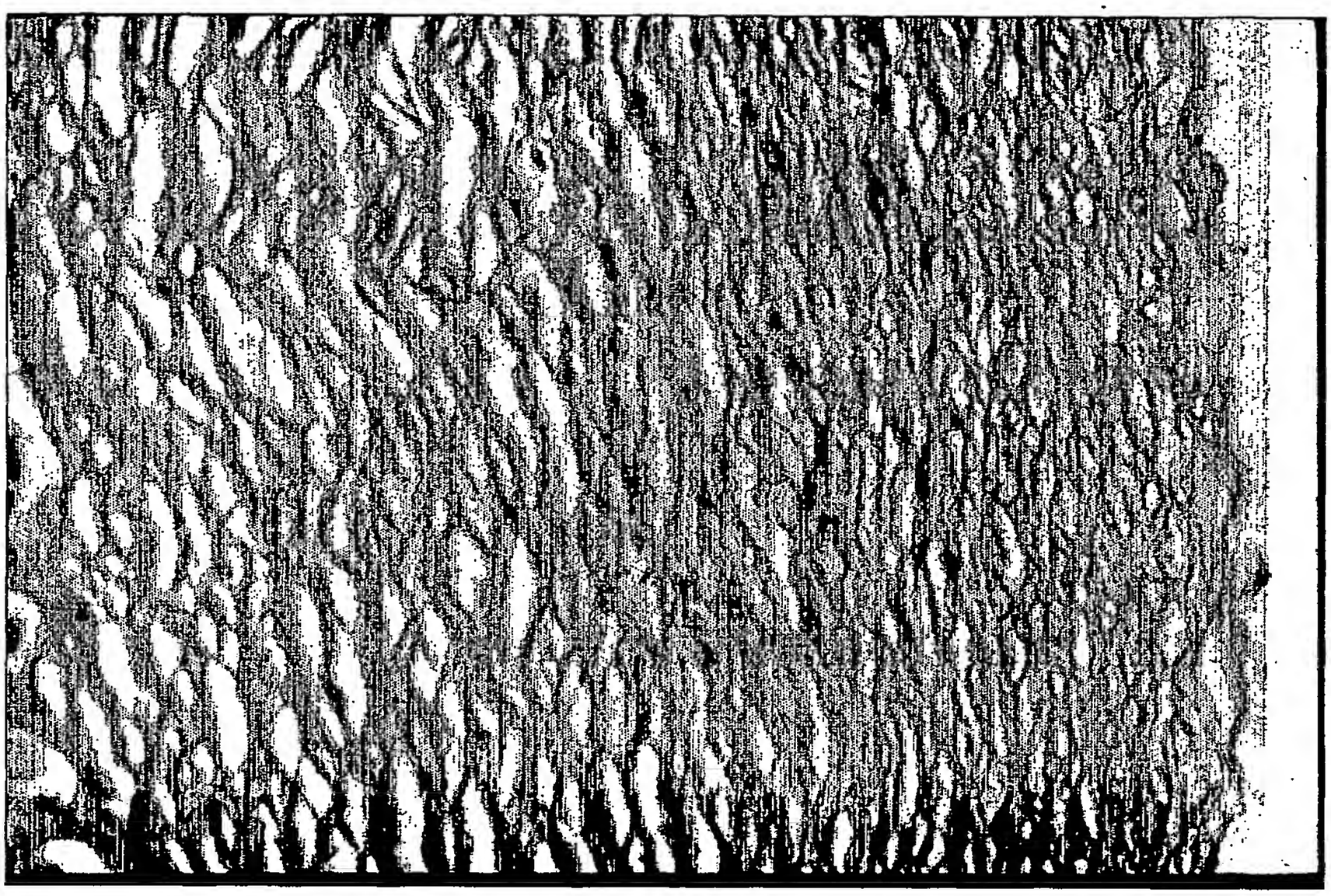

FIGURE 3B

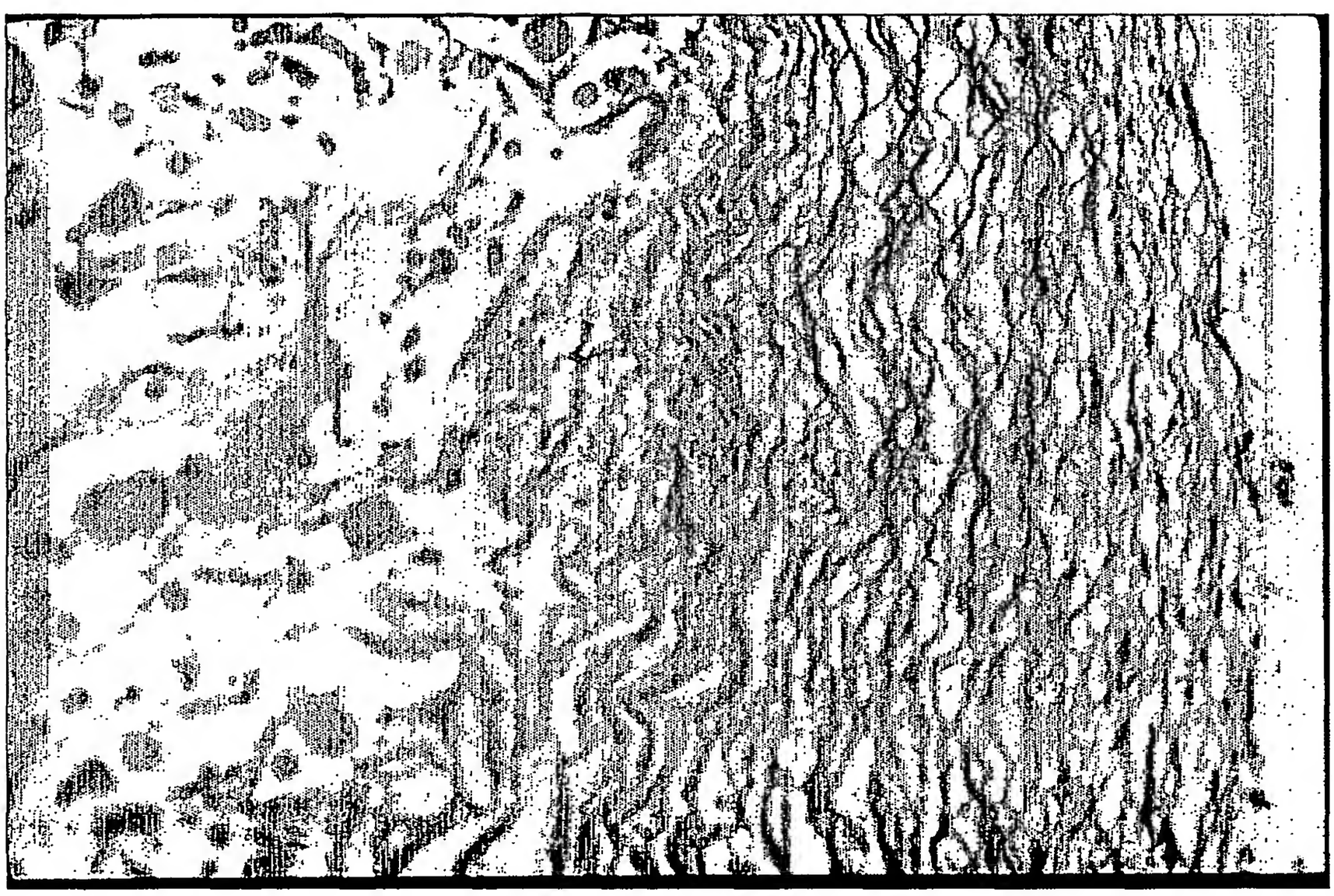

FIGURE 4A

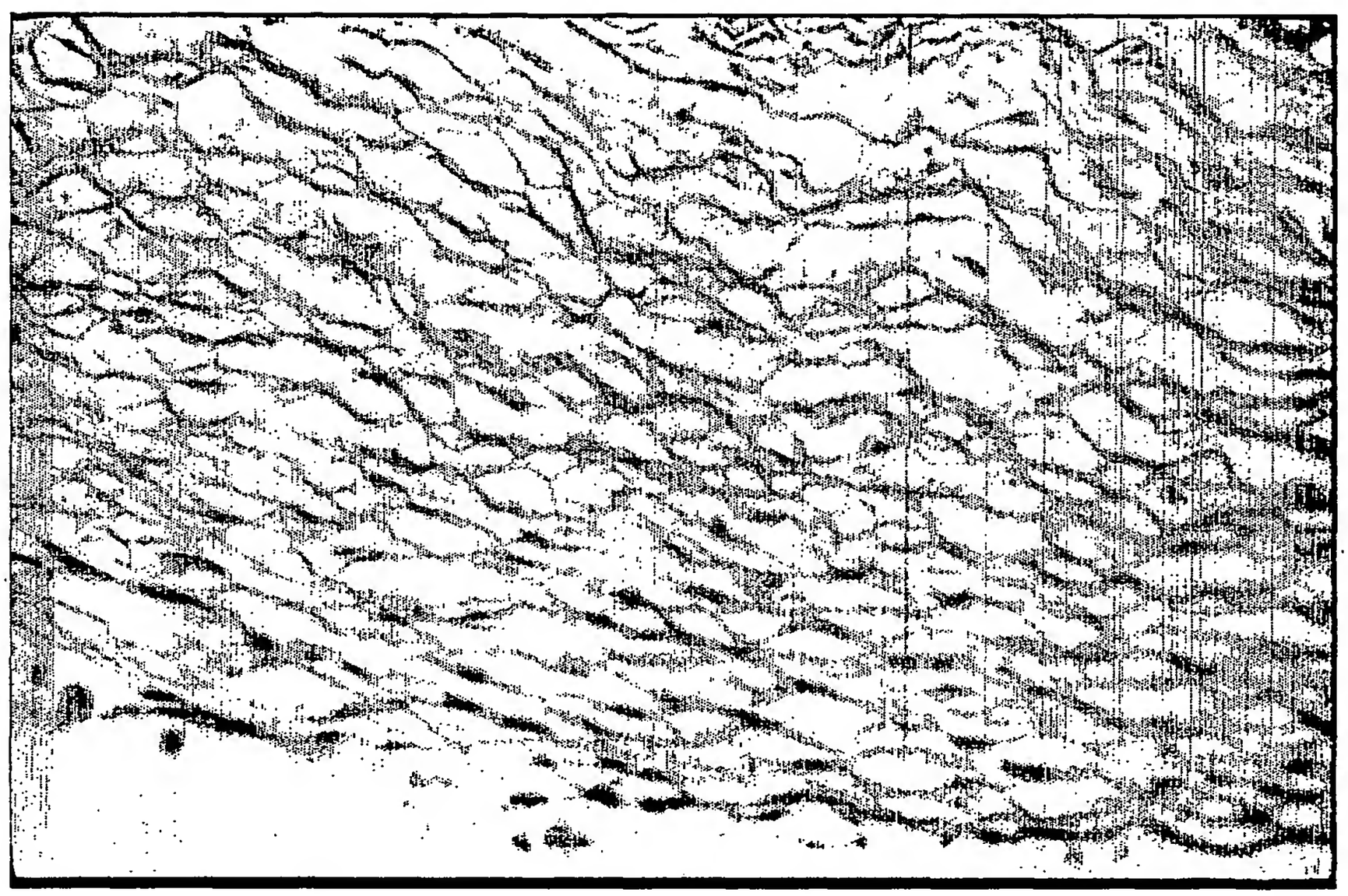

FIGURE 4B

FIGURE 5B

FIGURE 5A

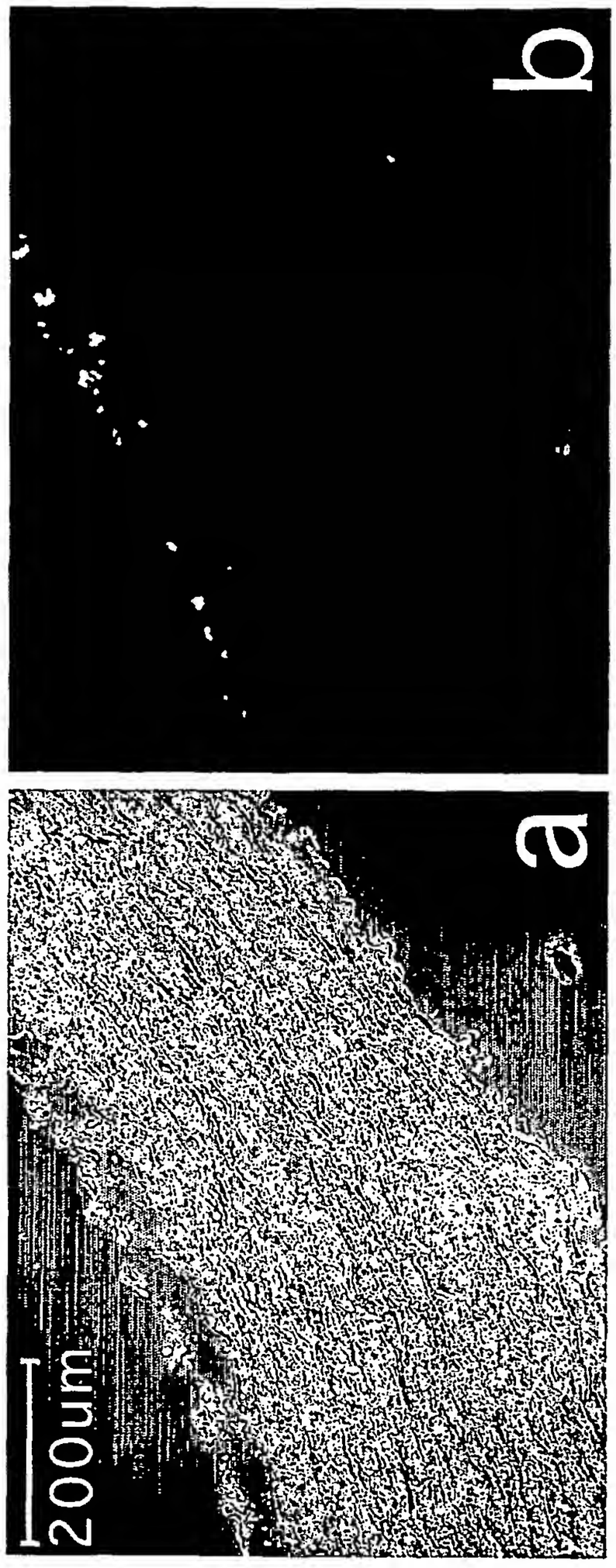

FIGURE 6A

FIGURE 6B

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(51) International Patent Classification[7]: C12N 5/06, A61L 27/28, 27/38, 27/50

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(71) Applicant *(for all designated States except US)*: **DUKE UNIVERSITY** [US/US]; 230 North Building, Research Drive, Durham, NC 27708 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MITCHELL, Shannon** [US/US]; 1717 Pergin Farm Road, Oakland, MD 21550 (US). **KOH, Jennifer** [US/US]; 11 Laurelglen, Irvin, CA 92614 (US). **NIKLASON, Laura, E.** [US/US]; 3301 Carriage Tr., Hillsborough, NC 27278 (US). **PRABHAKAR, Vikas** [US/US]; Durham, NC 27708 (US).

(74) Agent: **GERBER, Monica**; Choate, Hall & Stewart, Exchange Place, 53 State Street, Boston, MA 02109 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
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WO 02/014480 A3

(54) Title: DECELLULARIZED TISSUE ENGINEERED CONSTRUCTS AND TISSUES

(57) Abstract: New methods for producing tissue engineered constructs and engineered native tissues are disclosed. The methods include producing a tissue engineered construct bz growing cells *in vitro* on a substrate and then decellularizing the construct to produce a decellularized construct consisting largely of extracellular matrix components. The construct can bu used immediatley or stored unitl needed. The decellularized construct can be used for further tissue eingineering, which may include seeding the construct with cells obtained from the intended receipient of hte construct. During any of the growth phases required for production of the construct, the developing construct maz be subjected to various tissue engineering steps such as applciation of mechanical stimuli including pulsatile forces. The methods also include producing an engineered native tissue by harvesting tissue from an animal or human, performing one or more tissue engineering steps on the tissue, and subjecting the tissue to decellularization. The decellularized, engineered native tissue may then be subjected to further tissue engineering steps.

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 01/25628

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 A61L27/28 A61L27/38 A61L27/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NIKLASON L E ET AL: "Functional arteries grown in vitro." SCIENCE (WASHINGTON D C), vol. 284, no. 5413, 16 April 1999 (1999-04-16), pages 489-493, XP002198665 ISSN: 0036-8075 cited in the application the whole document --- -/--	1-6, 9-23,25, 27, 30-39, 57, 62-64, 67,82, 84,86, 90,101, 102,104, 110,122, 125,130, 132,146, 151,165, 168,173, 180,182, 205,210

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 May 2002	31/05/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mateo Rosell, A.M.

INTERNATIONAL SEARCH REPORT

Inter bnal Application No
PCT/US 01/25628

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 46165 A (CRYOLIFE INC) 22 October 1998 (1998-10-22) page 2, line 5-26 page 3, line 18 -page 7, line 9; example 1	1,16-18, 27, 30-39, 44-54, 59,61, 62, 82-84, 91,101, 125,132, 165,168, 174,182, 205,211, 218
X	US 5 613 982 A (GOLDSTEIN STEVEN) 25 March 1997 (1997-03-25) cited in the application column 4-16	1,2,22, 23,29, 30,39, 44,54, 57,59, 61,62, 67,68, 82,91, 96,101, 105,110, 121,130, 132,145, 165,168, 174,182, 192,195, 205,212, 218
X	HUYNH TAM ET AL: "Remodeling of an acellular collagen graft into a physiologically responsive neovessel." NATURE BIOTECHNOLOGY, vol. 17, no. 11, November 1999 (1999-11), pages 1083-1086, XP002198666 ISSN: 1087-0156 the whole document	1,6,7, 10-12, 15,18, 30,46, 59,60, 74,82, 84,90, 95,97, 101,104, 110,122, 130,132, 152,153, 162,165, 168,180, 182,183, 205,212, 217

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 01/25628

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	OBERPENNING F ET AL: "DE NOVO RECONSTITUTION OF A FUNCTIONAL MAMMALIAN URINARY BLADDER BYTISSUE ENGINEERING" NATURE BIOTECHNOLOGY, NATURE PUB. CO, NEW YORK, NY, US, vol. 17, no. 2, February 1999 (1999-02), pages 149-155, XP000986066 ISSN: 1087-0156 cited in the application the whole document ---	1,2,4-6, 9,12-14, 17,25, 30,57, 59, 61-63, 67-69, 82,93, 96,97, 101,105, 110,132, 151,154, 155,159, 168,174, 182,196, 205,211, 212,218
X	US 5 266 480 A (NAUGHTON GAIL K ET AL) 30 November 1993 (1993-11-30) cited in the application column 5, line 10-20 - line 55-60 column 10, line 60 -column 11, line 5 column 15, line 5-10 column 21, line 20 -column 55, line 30 ---	1,2,4-6, 9,12-14, 17,22, 23,25, 26,56, 57,59, 61, 67-69, 82,92, 95-97, 99,101, 105,110, 125,129, 132,151, 154,155, 159,161, 162,164, 168,174, 182,188, 191,192, 205,212
A	EP 0 528 039 A (IGAKI KEIJI) 24 February 1993 (1993-02-24) the whole document --- -/--	1,6,9, 14,15, 40-43

INTERNATIONAL SEARCH REPORT

Inte ronal Application No
PCT/US 01/25628

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCHMIDT C E ET AL: "Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering" BIOMATERIALS, ELSEVIER SCIENCE PUBLISHERS BV., BARKING, GB, vol. 21, no. 22, 15 November 2000 (2000-11-15), pages 2215-2231, XP004210279 ISSN: 0142-9612 the whole document ---	1
P,X	WO 01 49210 A (CHILDRENS MEDICAL CENTER) 12 July 2001 (2001-07-12) the whole document -----	1,2,17, 30, 32-39, 44-47, 49-57, 59, 61-63, 65,68, 82,87, 92,101, 105,110, 132,145, 168,174, 181,182, 205,212, 218

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-29, 32-61, 62-81, 82-109

In view of the large number and also the wording of the claims presently on file, which render it difficult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conciseness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and concise), namely the methods recited in the examples 1-7. Present claims 1-29, 32-61, 62-81, 82-109 (and their product claims: 110-131,132-167,132-167,168-181,182-204, 205-218), relate to a method defined by reference to a desirable characteristic or property, namely a method for producing a decellularized tissue engineered construct. An attempt is made to define the method by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the decellularized tissue engineered constructs prepared in examples 1-7.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/US 01/25628

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9846165 A	22-10-1998	AU 7105898 A	11-11-1998
		EP 0987998 A1	29-03-2000
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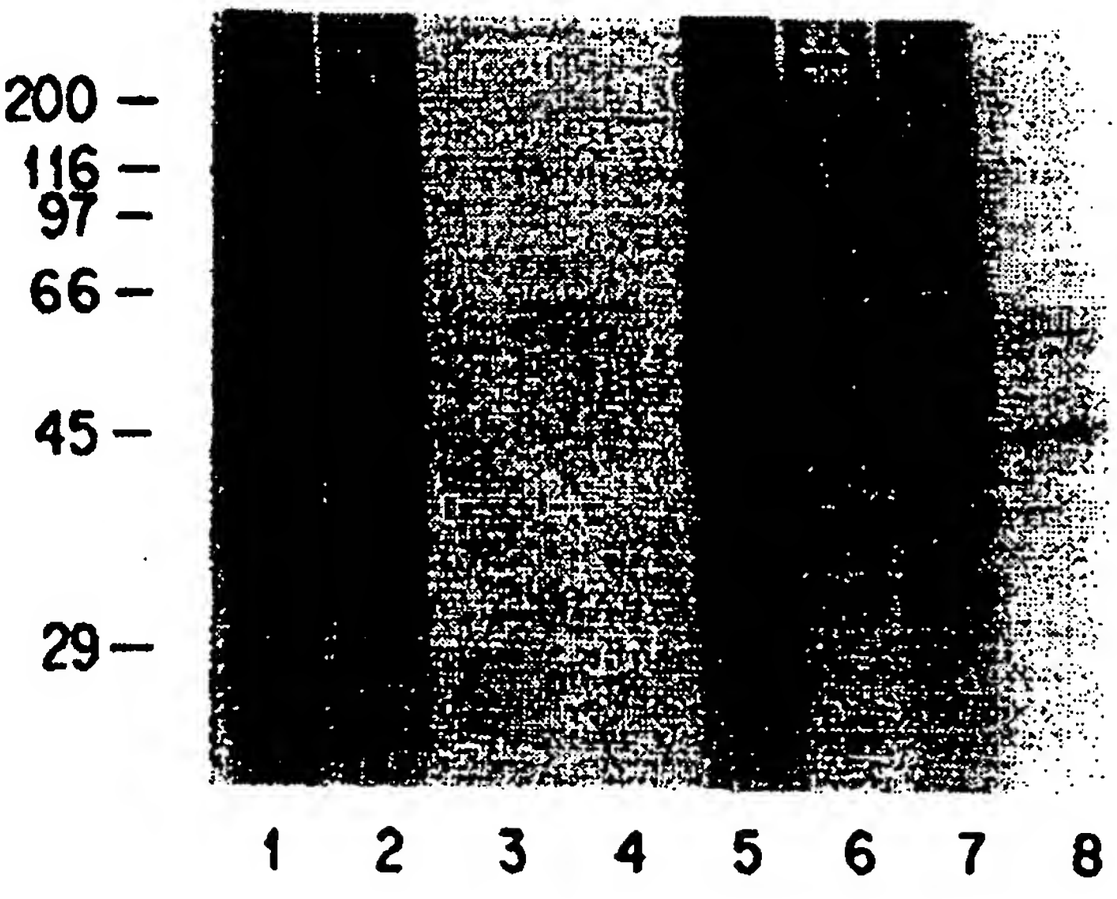
(54) Title: THREE-DIMENSIONAL HUMAN CELL CULTURES ON CARDIAC VALVE FRAMEWORKS AND THEIR USES

(57) Abstract

The present invention relates to a method of growing a variety of different cells and tissues in three-dimensional cultures *in vitro* using human fibroblasts in the culture medium. In accordance with the invention, stromal cells, including but not limited to human dermal and cardiac fibroblasts, are inoculated and grown on a three-dimensional scaffold or framework. The human fibroblasts secrete human matrix proteins to supplement and replace the existing porcine matrix composed of decellularized heart valves or aortic walls and leaflets formed into three-dimensional constructs having interstitial spaces bridged by the stromal cells. The living stromal tissue so formed provides the support, growth factors, and regulatory factors necessary to sustain long-term viability and proliferation of cells in culture and/or cultures implanted *in vivo*. When grown in this three-dimensional system, the proliferating cells mature and segregate properly to form components of tissues analogous to counterparts *in vivo*.



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THREE-DIMENSIONAL HUMAN CELL CULTURES ON CARDIAC VALVE FRAMEWORKS AND THEIR USES

1. INTRODUCTION

The invention relates to growing in vitro, human cells such as fibroblasts on a three-dimensional scaffold, comprising porcine aortic leaflets and walls, intact heart valves, other biological scaffolding suitable for reconstructing a valve or valve components, for example, including but not limited to the pericardium or the small intestinal submucosa, and biodegradable frameworks, such that the scaffold is populated with viable human cells having normal function, and fibroblasts are stimulated to produce human matrix proteins to supplement and replace the existing matrix on the scaffold.

The resulting three-dimensional tissue constructs have a variety of applications ranging from transplantation or implantation in vivo for replacement and/or reconstruction of a single valve component or the entire heart valve, to screening cytotoxic compounds and pharmaceutical compounds in vitro.

2. BACKGROUND OF THE INVENTION

Valve replacement surgical therapy is required for the treatment of various types of valvular heart diseases, including, but not limited to, aortic stenosis, aortic regurgitation, mitral stenosis, mitral regurgitation, pulmonary valve disease, tricuspid valve disease, multivalvular disease, Marfan syndrome and artificial valve disease. Two general types of valve replacement are available: the artificial, mechanical prosthesis or valve, and tissue biological prosthesis or valve. There are several kinds of mechanical prosthesis, such as the ball valve, the tilting disk and the central flow disk. There are also several tissue prostheses,

including preserved homografts and stent-mounted, porcine valve heterografts.

The primary advantage of the mechanical prosthesis is durability, whereas the disadvantage is a requirement that patients be on an anticoagulant therapy to reduce the risk of thromboembolic complications. This is because artificial mechanical heart valves are prone to occlusions by thrombus, and are subject to mechanical failures. Thromboembolism and anticoagulated hemorrhage are still the frequent causes for reoperation and patient death. Moreover, mechanical failure can occur suddenly and without warning resulting in emergency surgical interventions for replacement of the device. The advantage of the biological prothesis is a lower risk of thromboembolic complications, but may still require anticoagulant therapy in some situations. Moreover, the biological grafts which are currently used are not prone to sudden failure. However, the biological tissue grafts are also limited for a number of reasons. Allogeneic human valves (derived from a donor of the same species) are limited by the supply of donated human hearts and recipients must undergo immunosuppressive therapy to avoid rejection. While xenogeneic valves (derived from a donor of a different species) are more plentiful, they require treatment that results in calcification and a gradual degradation over time, requiring replacement. For example, allogeneic, transplanted heart valves may be obtained fresh, or may be cryopreserved to maintain viability of cellular components. Patients receiving allogeneic transplants usually must undergo immunosuppressive therapy. Despite such therapy, many of the transplants become inflamed and fail within five to ten years. Moreover, allogeneic valves are not as readily available as the xenogeneic valves. Xenogeneic biological valves, usually porcine or bovine in origin, have the advantage of being identical in design and structure to those valves being replaced, but are fixed

with glutaraldehyde and, therefore, are non-living. The glutaraldehyde-treated tissues calcify over time and do not allow infiltration and colonization by host cells, which is necessary for remodeling. Consequently, these xenogeneic valves degrade with time and eventually malfunction.

Autologous human tissue (i.e., derived from the recipient) is used for coronary and peripheral bypass procedures, third degree burns and reconstructive procedures involving bones and cartilage grafting. Such use eliminates complications of immunorejection resulting in better graft survival. Unfortunately, complications ensue with autologous heart valve transplants e.g., thrombosis and occlusion in the post-implant period and scarring of implant tissue. The development of alternative, tissue-based heart valves for transplantation is necessary due to unmet patient demands to improve upon existing heart valve technologies, which are mechanical valves requiring the constant use of anticoagulants and glutaraldehyde fixed tissue valves which eventually experience calcification.

Previous attempts at producing artificial tissues and organs have met limited success. Orton, U.S. Patent No. 5,192,312 describes treating a transplant tissue sample with exogenous basic fibroblast growth factor (BFGF) and repopulating the tissue with cells, preferably allogeneic or autogenous fibroblasts, ostensibly to avoid immunological rejection. The heart valves and heart leaflets may be sterilized with lethally effective doses of x-rays or with antibiotics, antibacterials and cytotoxic agents. According to Orton, the addition of bFGF to the tissue in vitro is critical in this system, and is essential for causing the graft-populating cells to migrate into the tissue and proliferate in response to the growth factor, and populate the tissue. However, Orton only demonstrates the system on small pieces of the valve fixed in a petri dish and does not show production

of a functional heart valve or any of the alleged advantages if implanted in vivo, e.g., avoidance of immunological rejection, and reduction in hardening and/or scarring of the valve transplant.

Livesey et al., U.S. Patent No. 5,336,616 relates to a method of producing a transplantable tissue graft for processing and preserving acellular, collagen-based tissue matrix for transplantation. The method described involved processing biological tissues with a stabilizing solution to prevent osmotic, hypoxic, autolytic and proteolytic degradation and to control contamination. The tissues were decellularized with EDTA, CHAPS or a zwitterionic detergent, SDS or anionic/nonionic detergent, followed by treatment with a cryoprotectant such as DMSO, propylene glycol, butanediol, raffinose, polyvinyl pyrrolidone, dextran or sucrose and vitrified in liquid nitrogen. Thereafter, the tissues were subjected to a dry stabilization procedure involving molecular distillation drying under nitrogen gas, followed by rehydration with buffered solution. Each of the methods has limitations and therefore it is essential that very stringent measures be taken to preserve the biological properties of the material and avoid toxicity resulting from reagents used during processing.

3. SUMMARY OF THE INVENTION

The present invention relates to transplantable cardiac tissue or bioprosthetic grafts composed of human cells grown on three-dimensional frameworks, scaffolds or matrices, a method of culturing human cells on such frameworks and uses of such three-dimensional cell cultures. In accordance with the invention, stromal cells, including but not limited to human fibroblasts, are inoculated and grown on a three-dimensional frameworks, such as intact heart valves, aortic walls and leaflets, or other biological scaffolding suitable for reconstructing a valve or valve components, including for

example, but not limited to the pericardium or the small intestinal submucosa or biodegradable frameworks or matrices. The preferred three-dimensional framework may be prepared from intact porcine heart valves, aortic wall tissue, or leaflets which are decellularized (at -20°C to -70°C or with detergents and enzymes) and sterilized by: chemical methods including, but not limited to, ethylene oxide and peracetic acid; irradiation including, but not limited to, gamma and electron beam; and steam sterilization including, but not limited to autoclaving. No viable cells remain in the decellularized/sterilized tissue samples which are used as a scaffold or framework for culturing the stromal cells.

The stromal cells which are inoculated onto the scaffold, may include dermal or cardiac fibroblasts, and/or cells capable of producing collagen types I and III, and in some instances, elastin, which are typically produced in heart valves. (See Table I). The stromal cells and connective tissue proteins naturally secreted by the stromal cells attach to and substantially envelope the three-dimensional framework or construct, having interstitial spaces bridged by the stromal cells. The living stromal tissue so formed provides the support, growth factors, and regulatory factors necessary to sustain long-term active proliferation of stromal cells in culture and/or cultures implanted in vivo. When grown in this three-dimensional system, the proliferating cells mature and segregate properly to form components of adult tissues analogous to counterparts in vivo.

In another embodiment of the invention, the stromal cells can be genetically engineered to express a gene product beneficial for successful and/or improved transplantation. For example, the stromal cells can be genetically engineered to express anticoagulation gene products to reduce the risk of thromboembolism, or anti-inflammatory gene products to reduce the risk of failure due to inflammatory reactions. For example, the stromal

cells can be genetically engineered to express tissue plasminogen activator (TPA), streptokinase or urokinase to reduce the risk of clotting. Alternatively, the stromal cells can be engineered to express anti-inflammatory gene products, e.g., peptides or polypeptides corresponding to the idiotype of neutralizing antibodies for tumor necrosis factor (TNF), interleukin-2 (IL-2), or other inflammatory cytokines. Preferably, the cells are engineered to express such gene products transiently and/or under inducible control during the post-operative recovery period, or as a chimeric fusion protein anchored to the stromal cell, e.g., a chimeric molecule composed of an intracellular and/or transmembrane domain of a receptor or receptor-like molecule, fused to the gene product as the extracellular domain.

In another alternative, the stromal cells can be genetically engineered to "knock out" expression of factors or surface antigens that promote clotting or rejection. For example, expression of fibrinogen, von Willebrands factor or any cell surface molecule that binds to the platelet $\alpha 2B\beta$-3 receptor can be knocked out in the stromal cells to reduce the risk of clot formation. Likewise, the expression of MHC class II molecules can be knocked out in order to reduce the risk of rejection of the graft.

In yet another embodiment of the invention, the three-dimensional culture system of the invention may afford a vehicle for introducing genes and gene products in vivo to assist or improve the results of the transplantation and/or for use in gene therapies. For example, genes that prevent or ameliorate symptoms of valvular disease such as thrombus formation, inflammatory reactions, fibrosis and calcification, may be underexpressed or overexpressed in disease conditions. Thus, the level of gene activity in the patient may be increased or decreased, respectively, by gene replacement

therapy by adjusting the level of the active gene product in genetically engineered stromal cells.

In a specific embodiment exemplified by the examples in Section 6, infra, human dermal fibroblasts were grown in the three-dimensional culture systems of the invention. Porcine aortic walls and leaflets were chosen because they are currently used in replacement therapy of heart valves. Particular benefits were achieved in porcine aortic wall and leaflet cultures where proliferation of human fibroblasts occurred, and production of tissue similar to human matrix proteins in the aortic walls and leaflets was detected. These characteristics were monitored by analyzing the recellularized or remodeled constructs for cell distribution (histological analyses), cell viability (MTT assays), cell proliferation (^{3}H-Thymidine labeling or BrdU incorporation), protein production (^{3}H-proline labeling, ^{35}S-cysteine/methionine labeling), and protein immunohistochemistry. The results from these studies showed that human dermal fibroblasts were able to colonize the porcine scaffolding of leaflets, aortic wall biopsies and intact valves, cultured and grown over several time intervals, for example, but not limited to 2, 4, 8 and 18 weeks. The present invention, thus, relates to a method of repopulating porcine aortic walls and leaflets with human fibroblasts to produce human matrix proteins in which the porcine aortic leaflets and walls are first sterilized with peracetic acid (or by other chemical means such as ethylene oxide) or by radiation with an electron beam (or by gamma irradiation) or by steam (autoclaving).

In the examples described infra, human fibroblasts were grown in culture on frameworks or constructs, composed of porcine aortic valves, walls and leaflets which had been decellularized and sterilized. When implanted in vivo, such frameworks or constructs allow adequate nutrient and gas exchange to the cells until

engraftment and vascularization at the site of engraftment occurs. The advantage of adding human fibroblasts to the three-dimensional, decellularized porcine scaffolds or biodegradable constructs, is that colonization of the porcine scaffolding results in a valve implant with living cells which produce biological factors that may stimulate host cells to endothelize the implant and stimulate host cardiac fibroblasts to integrate into the implant. The net result is enhancement of host-graft take. Another advantage of adding human fibroblasts is that cultures can be maintained under sterile conditions without inhibiting the growth of human fibroblasts, which grow in various types of frameworks or constructs usually pretreated with detergents or enzymes and sterilized with peracetic acid or irradiation with the electron beam. Furthermore, heart valves colonized with functional human cells are less likely to be subject to immunological rejection and thus are superior to those heart valves which are covered with xenogeneic cells prepared for use in replacement therapy.

It is an object of the present invention to construct a heart valve from human foreskin or cardiac fibroblasts and porcine heart valve and/or aortic walls and leaflets, which no longer contains porcine cells but becomes a humanized porcine heart valve or a recellularized heart valve suitable for transplantation in humans. Such an approach provides an improved method and means of designing, constructing and utilizing aortic walls and leaflets, intact heart valves other biological scaffolding suitable for reconstructing a valve or valve component (e.g., pericardium, small intestinal submucosa, etc.) and biodegradable frameworks, as scaffolding for growth and implantation of human fibroblasts in vitro.

It is further the object of the invention to construct a heart valve consisting of human cells and human tissue matrix proteins made by human dermal or

cardiac fibroblasts and a completely or nearly complete bioresorbable/biocompatible polymer scaffolding in the shape of different types of valves or their components, for example, but not limited to aortic, pulmonary, mitral, and tricuspid valves. Such an approach provides bioprosthetic or transplantable tissues, which can be utilized for cell growth, both in vitro and in vivo, to replace or reconstruct degenerated and dysfunctional heart valves in human patients.

It is a still further object of this invention to use human dermal or cardiac fibroblasts to colonize the porcine aortic leaflets and wall biopsies or other biological scaffolding suitable for reconstructing a valve or valve components (e.g., pericardium, small intestinal submucosa, etc.), and remain metabolically viable with the result that all porcine cells native to the leaflet and wall tissues or other biological scaffolds, are either eliminated (decellularized) or nonviable (dead). Such an approach provides an in vitro system in which human fibroblast cells retain their morphology and cell function for the secretion of bioactive molecules normally produced in the body by the cells of the aortic walls and leaflets or the intact heart valve or the pulmonary, mitral, and tricuspid valves.

The present invention relates to methods and biological tissue prothesis or valves for the treatment of valvular heart disease, including, but not limited to, aortic stenosis, aortic regurgitation, mitral stenosis, mitral regurgitation, pulmonary valve disease, tricuspid valve disease, multivalvular disease, tricuspid valve disease, Marfan syndrome and artificial valve disease.

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph of autoradiographed proteins synthesized by human dermal fibroblasts post seeding onto porcine aortic leaflets and walls.

Figure 2 is a photograph of hematoxylin and eosin stained tissue sections: a) a fresh porcine leaflet (the cardiac fibroblast nuclei native to the tissue appear purple in coloration); b) a detergent and/or enzyme extracted porcine leaflet (no porcine cell nuclei are detected after chemical treatment); c) a detergent and/or enzyme extracted porcine leaflet cultured with human fibroblasts for 18 weeks (the dermal human fibroblasts are present in the porcine matrix). (Stained with Hematoxylin/Eosin.) (10x).

Figure 3 is a photograph of a porcine leaflet seeded with human dermal fibroblasts and cultured for 4 weeks. (Stained with Hematoxylin/Eosin.)

Figure 4 is a bar graph showing that in three sample sets (#1-3) of detergent and/or enzyme extracted leaflets with or without fibroblasts, only the leaflets which are grown with fibroblasts incorporated ^{3}H-thymidine, indicating that the fibroblasts were proliferating.

Figure 5 is a SDS gel autoradiograph analysis showing protein bands: non-viable porcine leaflet (lane 1) and wall biopsy (lane 2) seeded with human fibroblasts show protein synthesis, whereas unseeded, non-viable porcine leaflet (lane 3) and porcine wall biopsy (lane 4) show no activity. Fresh, viable porcine leaflet (lane 5) and wall biopsy (lane 6) seeded with human fibroblasts have similar patterns to fresh, viable, unseeded porcine leaflet (lane 7) and wall biopsy (lane 8).

Figure 6. Porcine leaflet and wall (negative controls) a,b, respectively) stained with serum only and showed no background staining. Porcine leaflet stained with human tenascin (c) and porcine wall stained with human fibroblast antibody (d). Both c and d show no species cross reactivity. Whole humanized porcine valve

constructs cultured for 4 weeks under dynamic flow showed positive staining for human tenascin in the leaflet, wall, and muscle bar (e,g,i) and positive staining for human fibroblasts in the leaflet, wall, and muscle bar (f,h,j).

Figure 7 is a photograph of autoradiographed protein incorporation of human fibroblasts after dynamic culture on porcine aortic leaflets.

Figure 8 is a photograph depicting human fibroblast proliferation on a porcine matrix which was previously decellularized by detergent and/or enzyme treatment. The proliferating cells were labeled with Brdu and detected using an antibody to Brdu and a visualization kit. The labeled cells proliferating on the tissues were grown under dynamic flow conditions. Brdu labeling occurred during the last 72 hr of a 4 week culture period.

Figure 9 is a photograph depicting decellularized (detergent and/or enzyme) + electron beamed valves seeded with human fibroblasts under pulstile (left) and non-pulstile (right) dynamic flow conditions. Both valves were cultured dynamically for 1 week, then stained with MTT. The valve seeded with pulstile flow conditions had greater and more uniform fibroblast attachment.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to transplantable cardiac tissue constructs or bioprosthetic grafts grown in three-dimensional frameworks, a method of culturing human cells on such frameworks and uses of such three-dimensional, recellularized tissue constructs grown in cultures. In accordance with the invention, stromal cells, including but not limited to human fibroblasts, are inoculated and grown on a three-dimensional framework or construct of intact heart valves, aortic walls and

leaflets or other biological scaffolding suitable for reconstructing a valve or valve components, for example, including but not limited to the pericardium or the small intestinal submucosa or biodegradable frameworks. Cells grown on a three-dimensional framework, in accordance with the present invention, grow to form a cellular tissue-matrix which resembles tissue found in vivo to a greater degree than previously described. The three-dimensional cell culture system treated with human stromal cells is applicable to the proliferation of different types of cells and formation of a number of different tissues, including but not limited to aortic walls and leaflets, or intact heart valves, pulmonary, mitral, and tricuspid valves. In addition, the stromal cells grown in the system may be genetically engineered to produce gene products beneficial to transplantation, e.g., anti-coagulation factors, e.g., TPA, streptokinase, etc., or anti-inflammatory factors, e.g., anti-TNF, anti-IL-2, etc. Alternatively, the stromal cells may be genetically engineered to "knock out" expression of native gene products that promote platelet binding and clot formation, e.g., fibrinogen, von Willebrands factor, or "knock out" expression of MHC in order to lower the risk of rejection. In addition, the stromal cells may be genetically engineered for use in gene therapy to adjust the level of gene activity in a patient to assist or improve the results of the transplantation.

The use of human foreskin fibroblasts in the three-dimensional tissue constructs has a variety of advantages and applications. For example, for a variety of cells and tissues, such as porcine heart valves, aortic walls and leaflets, the chordae tendinea in the mitral and tricuspid valve, skin, ligaments, tendons, etc., the three-dimensional tissue constructs can be produced at a rapid rate and may itself be transplanted or implanted into a living organism without undue delay. The three-dimensional tissue constructs may also be used in vitro

for testing the effectiveness or cytotoxicity of pharmaceutical agents, screening compounds for use in treatment of clotting or thromboembolism, as anticoagulants, as anti-inflammatory agents, as anti-calcification agents or as endothelialization agents.

In yet another application, the three-dimensional tissue construct system may be cellularized within a "bioreactor" to produce a valve or valve component with leaflet mobility and full valve function. For example, an intact valve comprising of leaflets attached to the wall, may be assembled as a three-dimensional framework, inoculated with human stromal cells and maintained in recirculating culture medium regulated by a peristaltic or pneumatic pump which also keeps the leaflets or tissue sheets/patches in a dynamic state. The bioreactor provides a closed system free from problems of contamination during procedures involving sterilization, seeding, culturing, shipping and/or testing valve function.

The methods for culturing cells including human dermal fibroblasts on aortic walls and leaflet cells or intact heart valves or other biological scaffolding suitable for reconstructing a valve or valve components, for example, but not limited to the pericardium or the small intestinal submucosa or biodegradable frameworks, as a three-dimensional biological or synthetic framework or construct which can be used in accordance with the invention are described in applicants' co-pending application Serial No. 08/304,062 filed September 12, 1994; which is a continuation-in-part of Serial No. 08/254,096 filed June 6, 1994; which is a continuation-in-part of Serial No. 08/131,361 filed October 4, 1993, U.S. Patent No. 5,041,138 by Vacanti et al., and application Serial No. 07/509,952 filed April 16, 1990 by Vacanti et al., each of which is incorporated by reference herein in its entirety.

Methods for the treatment of valvular heart disease, including, but not limited to, aortic stenosis, aortic regurgitation, mitral stenosis, mitral regurgitation, pulmonary valve disease, tricuspid valve disease, multivalvular disease, tricuspid valve disease, Marfan syndrome and artificial valve disease, are described.

5.1. ESTABLISHMENT OF THREE-DIMENSIONAL FRAMEWORK

The three-dimensional framework for use in the present invention may be of any material and/or shape that: (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. It is preferred that allogeneic and xenogeneic aortic walls and leaflets or intact heart valves or other biological scaffolding suitable for reconstructing a valve or valve components, for example, but not limited to the pericardium or the small intestinal submucosa or biodegradable frameworks, obtained from a variety of mammals, including but not limited to, man, pig, cow, sheep or dog, may be used. The porcine leaflets and aortic biopsies may be used in the following forms: irradiated or chemically treated or steam treated (sterilized); decellularized (for example, detergent and/or enzyme treated), extracted and sterilized; and valve tissue with nonviable cells and other biological tissues, for example, but not limited to, pericardium or small intestinal submucosa (accomplished by such procedures as freezing at -20°C to -70°C, or by repeated freezing and thawing).

The methods for decellularizing the aortic walls and leaflets or intact valves or other biological scaffolding suitable for reconstructing a valve or valve components, which can be used in accordance with the invention, include, but are not limited to the methods described in U.S. Patent No. 5,336,616 and U.S. Patent No. 4,776,853, which are incorporated herein by reference in their

entirety. For example, the tissues can be decellularized with EDTA, CHAPS or a zwitterionic detergent, followed by treatment with a cryoprotectant such as DMSO, propylene glycol, butanediol, raffinose, polyvinyl pyrrolidone, dextran or sucrose and vitrified in liquid nitrogen. Alternatively, the tissue sample can be subjected to enzymatic digestion and/or extracting with reagents that break down the cellular membranes and allow removal of cell contents. Examples of detergents include non-ionic detergents (for example, TRITON X-100, octylphenoxy polyethoxyethanol, (Rohm and Haas); BRIJ-35, a polyethoxyethanol lauryl ether (Atlas Chemical Co.), TWEEN 20, a polyethoxyethanol sorbitan monolaureate (Rohm and Haas), LUBROL-PX, or polyethylene lauryl ether (Rohm and Haas)); and ionic detergents (for example, sodium dodecyl sulphate, sulfated higher aliphatic alcohol, sulfonated alkane and sulfonated alkylarene containing 7 to 22 carbon atoms in a branched or unbranched chain). The enzymes used may include nucleases (for example, deoxyribonuclease and ribonuclease), proteases, phospholipases and lipases. The tissues in the invention can also be decellularized using physical procedures such as ultrasonic treatment or osmotic shock, or by chemical treatment using peracetic acid.

The three-dimensional framework may also be composed of completely or nearly complete bioresorbable/ biocompatible polymer scaffolding in the shape of various different types of valves, including but not limited to, aortic, pulmonary, mitral, and tricuspid valves and valve components of each type. The biodegradable scaffolds, constructs, frameworks or matrices may be composed of materials such as polyglycolic acid, catgut suture material, hyaluronic acid, cellulose, collagen (in the form of sponges, braids, or woven threads, etc.), gelatin, or other naturally occurring biodegradable materials or synthetic materials, including for example, a variety of polyhydroxyalkanoates. Such frameworks or

constructs may be molded into the shape of heart valves or repair sheets/patches prior to inoculation of human cells. Where possible, however, it is most preferable to use a three-dimensional construct of the tissue of origin, for example, the aortic walls and leaflets or intact heart valves.

The invention is based in part, on the discovery that the three-dimensional system supports the proliferation, migration, differentiation, and segregation of cells in culture in vitro to form components of tissues analogous to counterparts found in vivo. The human cells added to the scaffolds repopulate the porcine valve without the need for exogenously added growth factors. This is contrary to Orton's teachings which show that leaflet tissue not treated with bFGF remained acellular. The use of growth factors (for example, but not limited to, αFGF, βFGF, insulin growth factor or TGF-betas), or natural or modified blood products or other bioactive biological molecules (for example, but not limited to, hyaluronic acid or hormones), even though not absolutely necessary in the present invention, may be used to further enhance the reconstitution of the porcine or other biological scaffolding.

Although the applicants are under no duty or obligation to explain the mechanism by which the invention works, a number of factors inherent in the three-dimensional culture system may contribute to its success:

(a) The three-dimensional framework provides a greater surface area for protein attachment, and consequently, for the adherence of stromal cells.

(b) Because of the three-dimensionality of the framework, stromal cells continue to actively grow, in contrast to many cells in monolayer cultures, which grow to confluence, exhibit contact inhibition, and cease to grow and divide. The elaboration of extracellular matrix

proteins and secretion of growth and regulatory factors by replicating stromal cells may be partially responsible for stimulating proliferation, maintaining normal tissue differentiation and regulating differentiation of cells in culture.

(c) The three-dimensional framework allows for a spatial distribution of cellular elements which is more analogous to that found in the counterpart tissue in vivo.

(d) The increase in potential volume for cell growth in the three-dimensional system may allow the establishment of localized microenvironments conducive to cellular maturation.

(e) The three-dimensional framework maximizes cell-cell interactions by allowing greater potential for movement of migratory cells.

(f) It has been recognized that maintenance of a differentiated cellular phenotype requires not only growth/differentiation factors but also the appropriate cellular interactions. The present invention effectively recreates the tissue microenvironment.

The three-dimensional stromal support, the culture system itself, and its maintenance, as well as various uses of the three-dimensional cultures are described in greater detail in the subsections below.

5.2. ESTABLISHMENT OF THREE-DIMENSIONAL STROMAL TISSUE

Stromal cells comprising fibroblasts, with or without other stromal cells and elements described below, are inoculated onto the three-dimensional framework. Human fibroblasts may be added to the culture prior to, during or subsequent to inoculation of other stromal cells. The concentration of fibroblasts maintained in the cultures can be monitored and adjusted appropriately to optimize growth and to regulate scaffold colonization. Alternatively, stromal cells that are genetically

engineered to express and produce factors similar to those produced by cells of the heart valve, may be included in the inoculum. These cells could serve as a source of protein factor(s) in the culture. Preferably, the gene or coding sequence for factor(s) would be placed under the control of a regulated promoter, so that production of factor(s) in culture can be controlled. The genetically engineered cells will be screened to select those cell types: 1) that bring about amelioration of blood clotting, coagulation, thromboembolism and inflammatory reactions in vivo, and 2) escape immunological surveillance and rejection.

Stromal tissue comprising dermal fibroblasts, cardiac fibroblasts and cells capable of producing collagen type I and III, elastin and other heart valve matrix proteins, for example, but not limited to fibronectin and glycosaminoglycans, are used to grow in vitro, transplantable tissue or bioprosthetic heart valves. Stromal cells such as fibroblasts can be obtained in quantity rather conveniently from skin, human foreskin, heart tissue or any appropriate organ. Fetal and neonatal fibroblasts can be used to form a "generic" three-dimensional stromal tissue construct that will support the growth of a variety of different cells and/or tissues. Fibroblasts may be readily isolated by disaggregating an appropriate organ or tissue which is to serve as the source of the fibroblasts. This may be readily accomplished using techniques known to those skilled in the art. For example, the tissue or organ can be disaggregated mechanically and/or treated with digestive enzymes and/or chelating agents that weaken the connections between neighboring cells making it possible to disperse the tissue into a suspension of individual cells without appreciable cell breakage. Enzymatic dissociation can be accomplished by mincing the tissue and treating the minced tissue with any of a number of digestive enzymes either alone or in combination. These

include but are not limited to trypsin, chymotrypsin, collagenase, elastase, hyaluronidase, pronase, etc. Mechanical disruption can also be accomplished by a number of methods including, but not limited to the use of grinders, blenders, sieves, homogenizers, or pressure cells to name but a few. For a review of tissue disaggregation techniques, see Freshney, Culture of Animal Cells. A Manual of Basic Technique, 2d Ed., A.R. Liss, Inc., New York, 1987, Ch. 9, pp. 107-126.

Once the tissue has been reduced to a suspension of individual cells, the suspension can be fractionated into subpopulations from which the fibroblasts and/or other stromal cells and/or elements can be obtained. This also may be accomplished using standard techniques for cell separation including but not limited to cloning and selection of specific cell types, selective destruction of unwanted cells (negative selection), separation based upon differential cell agglutinability in the mixed population, freeze-thaw procedures, differential adherence properties of the cells in the mixed population, filtration, conventional and zonal centrifugation, centrifugal elutriation (counter-streaming centrifugation), unit gravity separation, counter current distribution, electrophoresis and fluorescence-activated cell sorting. For a review of clonal selection and cell separation techniques, see Freshney, Culture of Animal Cells. A Manual of Basic Techniques, 2d Ed., A.R. Liss, Inc., New York, 1987, Ch. 11 and 12, pp. 137-168.

The isolation of fibroblasts may, for example, be carried out as follows: fresh tissue samples are thoroughly washed and minced in Hanks balanced salt solution (HBSS) in order to remove serum. The minced tissue is incubated from 1-12 hours in a freshly prepared solution of a dissociating enzyme such as trypsin. After such incubation, the dissociated cells are suspended, pelleted by centrifugation and plated onto culture

dishes. Fibroblasts will attach before other cells, therefore, appropriate stromal cells can be selectively isolated and grown. The isolated fibroblasts can then be grown to confluency, lifted from the confluent culture and inoculated onto the three-dimensional support (see, Naughton et al., 1987, J. Med. 18(3&4):219-250). Inoculation of the three-dimensional matrix with a high concentration of stromal cells, e.g., approximately 10^6 to 5×10^7 cells/ml, will result in the establishment of the three-dimensional stromal construct in shorter periods of time.

Again, where the cultured cells are to be used for transplantation or implantation in vivo it is preferable to obtain the stromal cells from the patient's own tissues. However, it is also possible to use allogeneic compatible human cells, without significant rejection reactions following transplantation. The growth of cells in the presence of the three-dimensional stromal support matrix may be further enhanced by adding to the matrix, or coating the matrix support with specific amino acids, proteins, glycoproteins, glycosaminoglycans, a cellular matrix, and/or other materials.

After inoculation of the stromal cells, the three-dimensional matrix should be incubated in an appropriate nutrient medium. Many commercially available media such as DMEM, RPMI 1640, Fisher's Iscove's, McCoy's, and the like may be suitable for use. It is preferable that the three-dimensional stromal matrix be suspended or floated in the medium during the incubation period in order to maximize proliferative activity. The container in this protocol is kept stable in the incubator, i.e., under static conditions (no circulating or flowing fluid). In addition, the culture should be "fed" periodically to remove the spent media, depopulate released cells, and add fresh nutrients. The concentration of fibroblasts may be adjusted during these steps. These procedures are greatly facilitated when carried out using a bioreactor,

which is a closed system housing the three-dimensional framework inoculated with stromal cells. A bioreactor reduces the possibility of contamination, maintains the cultures in recirculating, continuous culture medium and keeps the leaflets in a dynamic state by opening and closing them. More particularly, the U.S. patent application entitled "Apparatus and Method for Sterilizing, Seeding, Culturing, Storing, Shipping, and Testing Tissue, Synthetic or Mechanical Heart Valves or Valve Segments" and filed concurrently herewith by the assignee of the present application, teaches the mode of operation of the bioreactor and, is incorporated by reference herein.

During the incubation period, the stromal cells will attach and proliferate along the three-dimensional framework before beginning to migrate into the depths of the matrix. One objective is to grow the cells to an appropriate degree which reflects the amount of stromal cells present in the <u>in vivo</u> tissue. A second objective is to regulate the number of cells in the inoculum and/or their growth on the scaffold such that the amount of scaffold colonization can be controlled as desired, and reproducibly.

The openings of the non-tissue framework or constructs should be of an appropriate size to allow the stromal cells to stretch across the openings. Maintaining actively growing stromal cells which stretch across the framework enhances the production of growth factors which are elaborated by the stromal cells, and hence will support long term cultures. For example, if the openings are too small, the stromal cells may rapidly achieve confluence but be unable to easily exit from the mesh; trapped cells may exhibit contact inhibition and cease production of the appropriate factors necessary to support proliferation and maintain long term cultures. If the openings are too large, the stromal cells may be unable to stretch across the opening; this will also

decrease stromal cell production of the appropriate factors necessary to support proliferation and maintain long term cultures. When using a mesh type of matrix, as exemplified herein, we have found that openings ranging from about 150 μm to about 220 μm will work satisfactory. However, depending upon the three-dimensional structure and intricacy of the framework, other sizes may work equally well. In fact, any shape or structure that allows the stromal cells to stretch and continue to replicate and grow for lengthy time periods will work in accordance with the invention.

The human dermal fibroblasts exhibit a varied affinity for the different types of porcine tissue matrices. The greatest fibroblast colonization occurs when using a porcine matrix that is detergent and/or enzyme extracted. Additionally, the amount of fibroblast colonization in the porcine tissue correlates with time.

Different proportions of the various types of collagen deposited on the framework can be manipulated. For example, for optimal growth of transplantable or bioprosthetic heart valves, collagen types I and III are preferably deposited in the initial matrix. The proportions of collagen types deposited can be manipulated or enhanced by selecting fibroblasts or cells which elaborate the appropriate collagen type. This can be accomplished using monoclonal antibodies of appropriate isotypes or subclass that are capable of activating complement, and which define particular collagen type. .These antibodies and complement can be used to negatively select the fibroblasts which express the desired collagen type. Alternatively, the stroma used to inoculate the matrix can be a mixture of cells which synthesize the appropriate collagen types desired. The distribution and origins of the five types of collagen is shown in Table I. Thus, for the growth and preparation of heart valves, fibroblasts are the preferred cells for the present invention.

TABLE I

DISTRIBUTIONS AND ORIGINS OF
THE FIVE TYPES OF COLLAGEN

	Tissue Distribution	Cells of Origin
I	connective tissue; collagen fibers	reticular cells; smooth muscle cells
	Fibrocartilage	
	Bone	Osteoblast
	Heart Valve	Fibroblasts
	Dentin	Odontoblasts
II	Hyaline and elastic cartilage	Chondrocytes
	Vitreous body of eye	Retinal cells
III	Loose connective tissue; reticular fibers	Fibroblasts and reticular cells
	Papillary layer of dermis	
	Blood vessels	Smooth muscle cells; endothelial cells
IV	Basement membranes	Epithelial and endothelial cells
	Lens capsule of eye	Lens fibers
V	Fetal membranes; placenta	Fibroblast
	Basement membranes	

Bone	
Smooth muscle	Smooth muscle cells

During incubation of the three-dimensional stromal construct, proliferating cells may be released from the framework. These released cells may stick to the walls of the culture vessel where they may continue to proliferate and form a confluent monolayer. This should be prevented or minimized, for example, by removal of the released cells during feeding, by coating the culture vessel with substances such as silicone to decrease cellular attachment, or by transferring the three-dimensional stromal framework to a new culture vessel. The presence of a confluent monolayer in the vessel will "shut down" the growth of cells in the three-dimensional framework and/or culture. Removal of the confluent monolayer or transfer of the framework to fresh media in a new vessel will restore proliferative activity of the three-dimensional culture system. Such removal or transfers should be done in any culture vessel which has a stromal monolayer exceeding 25% confluency.

Alternatively, the culture system could be agitated to prevent the released cells from adhering, or instead of periodically feeding the cultures, the culture system could be set up so that fresh media continuously flows through the system. The flow rate could be adjusted to both maximize proliferation within the three-dimensional culture, and to wash out and remove cells released from the matrix, so that they will not adhere to the walls of the vessel and grow to confluence.

5.3. USES OF THE TRANSPLANTABLE HUMAN CELL-COLONIZED HEART VALVES AND SHEETS GROWN IN THREE-DIMENSIONAL CULTURE SYSTEM

The three-dimensional culture system of the invention can be used in a variety of applications. These include but are not limited to transplantation or implantation of either the cultured tissue obtained from the framework, or the cultured matrix itself *in vivo*; screening the effectiveness and cytotoxicity of pharmaceutical agents, blood related natural and modified compounds, growth/regulatory factors, etc., *in vitro*; elucidating the mechanism of certain diseases; studying the mechanism by which drugs and/or growth factors operate; gene therapy; and the production of biologically active products, to name but a few.

5.3.1. TRANSPLANTATION IN VIVO

The biological heart valves produced in the three-dimensional culture system of the invention can be used in the treatment of aortic stenosis, aortic regurgitation, mitral stenosis, mitral regurgitation, pulmonary valve disease, tricuspid valve disease, multivalvular disease, tricuspid valve disease, Marfan syndrome and artificial valve disease.

Aortic stenosis is the obstruction to flow across the aortic valve during left ventricular systolic ejection. It can be caused by a congenital unicuspid or bicuspid valve, rheumatic fever, or degenerative calcification of the valve in the elderly. The incidence of bicuspid aortic valve has been estimated at 4 in 1,000 live births, with males dominating over females at 4:1. Campbell, M., and Kauntze, R., 1953, Br. Heart J. 15:179. Leaflets often thicken by age 40 and almost invariably by age 50, but calcium deposits are rarely detected before 40 years of age. Although symptoms generally occur late in the course of aortic stenosis, 3 to 5 percent of patients may die suddenly during an otherwise a

symptomatic period. Thus, patients with any sign of congestive heart failure, angina, or exertional syncope in the presence of significant aortic valvular stenosis should undergo aortic valve replacement promptly. In addition, asymptomatic patients with significant aortic valvular stenosis should be advised to have valve replacement therapy.

Aortic regurgitation is the diastolic flow of blood from the aorta into the left ventricle. It is caused by incompetent closure of the aortic valve which results from intrinsic disease of the cusp or from diseases affecting the aorta. Acquired intrinsic diseases of the aortic valve are either rheumatic or from bacterial origin. In the Marfan syndrome the primary basis for aortic insufficiency usually resides in the aorta, but there may be prolapse of the aortic cusps due to myxomatous changes. Infrequent changes are seen with rheumatoid arthritis, systemic lupus erythematosus, and trauma. Oh, W.M.C., Taylor, T.R. and Olsen, E.G.J., 1974, Br. Heart J. 36:413. Patients with chronic aortic regurgitation who are symptomatic are advised to have surgery. The type of operation used depends primarily on the etiology. In patients with diseases limited to the valve, the operation is essentially as described above, for aortic stenosis.

Mitral stenosis designates resistance to flow through the mitral apparatus during diastolic filling of the left ventricle. Resistance to diastolic flow across the mitral valve can result from rheumatic valvulitis, congenital stenosis, thrombus formation, atrial myxoma, bacterial vegetations, and calcification in the valve, as well as in the annulus. The decision to intervene surgically in patients with mitral stenosis is based on the anticipated necessity of valve replacement versus valve reconstruction therapy.

Mitral regurgitation occurs when contraction of the left ventricle ejects blood into left atrium as a result

of abnormalities in the mitral valve apparatus. Acute mitral regurgitation can be created from mechanical disruption of the chordae tendineae, rupture of the papillary muscle, or perforation of the leaflet. Rheumatic fever, mitral valve prolapse and coronary artery disease, such as left ventricular dilation, calcified mitral annulus, heritable disorders (Marfan syndrome, Ehlers-Danlos, osteogenesis), congenital heart disease, systemic lupus erythematosus, rupture of papillary muscle and perforation of leaflet, are the predominant mechanisms for the incompetence of the mitral valve. Replacement of the mitral valve, valve components and/or other affected parts such as the chordae, is required in cases of rheumatic involvement leading to severe mitral regurgitation, mitral stenosis with loss of pliability of the leaflets, and various other causes of mitral regurgitation, such as infective endocarditis, and in some cases in chronic heart disease. Calcification and immobility of the leaflets are also indications for valve replacement.

Pulmonary stenosis is created by obstruction to systolic flow across the valve and is most commonly congenital. It generally leads to pulmonary regurgitation. Pulmonary valve replacement may be performed for acquired conditions such as carcinoid heart disease and infective endocarditis.

Tricuspid regurgitation develops when the tricuspid valve allows blood to enter the right atrium during right ventricular contraction. Tricuspid stenosis represents obstruction to diastolic flow across the valve during diastolic filling of the right ventricle. The main cause of tricuspid and mitral regurgitation is the rupture of one or more of the elements of the tensor apparatus, with disruption of the papillary muscle and rupture of the chordae tendineae. Replacement is necessary if the changes in the leaflets and subvalvular mechanism are

advanced, or if severe regurgitation cannot be relieved by annulopolasty.

Multivalvular disease indicates obstruction and/or incompetence of the aortic, mitral, and tricuspid valves. Rheumatic fever, connective tissue diseases, Marfan syndrome, calcification of the mitral valve in the aging patient and bacterial endocarditis remain important causes in combined disease of the mitral and aortic valves. In patients with severe and progressive symptoms having evidence of disease at both the mitral and aortic valves, both valves are generally repressed by surgery.

Artificial valve disease includes any abnormality of a surgically implanted device to replace a diseased cardiac valve. Artificial valve disease can result from prosthetic dysfunction, thrombus formation, infection, fibrosis, or calcification. Roberts, W.C., 1973, Prog. Cardiovasc. Dis. 15:539. Congestive heart failure due to mechanical valve dysfunction is the major indication for replacement of a mechanical artificial valve. Replacement of the prosthesis is indicated if the symptoms cannot be controlled medically or if there is evidence of progressive ventricular dysfunction.

The second most common operation performed in adults is replacement of the aortic or mitral valve. The valves produced in accordance with the invention may be transplanted using similar, if not the same surgical techniques, well known to those skilled in the art. The procedure for the replacement of the aortic valve is performed through a median sternum-splitting incision. After cardiopulmonary bypass is begun, a vascular clamp is placed across the distal ascending aorta. A sump suction cannula is placed in the left atrium through an incision in the right superior pulmonary vein to decompress the left heart. A transverse incision is made in the proximal aorta and the diseased valve is excised. Horizontal mattress sutures are placed at the three commissures for traction. Simple radial sutures are then

placed along the annulus between traction sutures and passed sequentially through the sewing ring of the valve as they are inserted. When all sutures have been passed through the sewing ring, the valve is lowered into position and the sutures are tied and cut. The aortotomy is closed with continuous sutures.

Coronary artery perfusion usually is not necessary for single-valve replacement, provided it can be accomplished in 60 minutes or less. Adequate myocardial protection can be afforded by systemic hypothermia at 30·C, injection of cardioplegic solution into the ascending aorta, and lavage of the heart by iced isotonic solution before it is opened.

In case of a mitral valve replacement, tricuspid valve replacement or a pulmonary valve replacement, the procedure is modified accordingly and involves the same technical maneuvers as outlined above. A detailed description of the operative surgery used is described in P.F. Nora, ed., Operative Surgery Principles and Techniques, 2nd ed. (1980) 326-327; J.W. Kirklin and B.G. Barratt-Boyes, eds., Cardiac Surgery Morphology, Diagnostic Criteria, Natural History, Techniques, Results, and Indications, 2d ed. (1993) 498-507; and J.W. Hurst and R.C. Schlant, eds., The Heart, Arteries and Veins, 7th ed. (1990) 795-876.

For transplantation or implantation in vivo, either portions of the culture or the entire three-dimensional culture could be implanted, depending upon the type of tissue involved. For example, three-dimensional heart valve cultures can be maintained in vitro for long periods. Section of tissues or the entire three-dimensional tissue structure can be transplanted in vivo in patients needing new heart valves.

Three-dimensional tissue culture implants may, according to the inventions, be used to replace or augment existing tissue, to introduce new or altered

tissue, to modify artificial prostheses, or to join together biological tissues or structures.

5.3.2. SCREENING EFFECTIVENESS AND CYTOTOXICITY OF COMPOUNDS IN VITRO

The three-dimensional cultures may be used in vitro to screen a wide variety of compounds, for effectiveness and cytotoxicity of pharmaceutical agents, growth/regulatory factors, natural and modified blood products, anticoagulants, clotting agents or anti-calcification agents, etc. To this end, the cultures are maintained in vitro and exposed to the compound to be tested. The activity of a cytotoxic compound can be measured by its ability to damage or kill cells in culture. This may readily be assessed by vital staining techniques. The effect of growth/regulatory factors may be assessed by analyzing the cellular content of the matrix, e.g., by total cell counts, and differential cell counts. This may be accomplished using standard cytological and/or histological techniques including the use of immunocytochemical techniques employing antibodies that define type-specific cellular antigens. The effect of various drugs on normal cells cultured in the three-dimensional system may be assessed.

5.3.3. GENE THERAPY

The three-dimensional culture system of the invention may afford a vehicle for introducing genes and gene products in vivo to assist or improve the results of the transplantation and/or for use in gene therapies. For example, the stromal cells can be genetically engineered to express anticoagulation gene products to reduce the risk of thromboembolism, or anti-inflammatory gene products to reduce the risk of failure due to inflammatory reactions. In this regard, the stromal cells can be genetically engineered to express TPA, streptokinase or urokinase to reduce the risk of

clotting. Alternatively, the stromal cells can be engineered to express anti-inflammatory gene products, for example, peptides or polypeptides corresponding to the idiotype of neutralizing antibodies for TNF, IL-2, or other inflammatory cytokines. Preferably, the cells are engineered to express such gene products transiently and/or under inducible control during the post-operative recovery period, or as a chimeric fusion protein anchored to the stromal cells, for example, a chimeric molecule composed of an intracellular and/or transmembrane domain of a receptor or receptor-like molecule, fused to the gene product as the extracellular domain. In another embodiment, the stromal cells could be genetically engineered to express a gene for which a patient is deficient, or which would exert a therapeutic effect, e.g., HDL, apolipoprotein E, etc. The genes of interest engineered into the stromal cells need to be related to heart disease. For example, the stromal cells can be engineered to express gene products that are carried by the blood; e.g., cerebredase, adenosine deaminase, α-1-antitrypsin. In a particular embodiment, a genetically engineered valve culture implanted to replace the pulmonary valve can be used to deliver gene products such as α-1-antitrypsin to the lungs; in such an approach, constitutive expression of the gene product is preferred.

The stromal cells can be engineered using a recombinant DNA construct containing the gene used to transform or transfect a host cell which is cloned and then clonally expanded in the three-dimensional culture system. The three-dimensional culture which expresses the active gene product, could be implanted into an individual who is deficient for that product. For example, genes that prevent or ameliorate symptoms of various types of valvular heart diseases may be underexpressed or down regulated under disease conditions. Specifically, expression of genes involved in preventing the following pathological conditions may

be down-regulated, for example: thrombus formation, inflammatory reactions, and fibrosis and calcification of the valves. Alternatively, the activity of gene products may be diminished, leading to the manifestations of some or all of the above pathological conditions and eventual development of symptoms of valvular disease. Thus, the level of gene activity may be increased by either increasing the level of gene product present or by increasing the level of the active gene product which is present in the three-dimensional culture system. The three-dimensional culture which expresses the active target gene product can then be implanted into the valvular disease patient who is deficient for that product. "Target gene," as used herein, refers to a gene involved in valvular disease in a manner by which modulation of the level of target gene expression or of target gene product activity may act to ameliorate symptoms of valvular disease.

Further, patients may be treated by gene replacement therapy during the post-recovery period after transplantation. Heart valve constructs or sheets may be designed specifically to meet the requirements of an individual patient, for example, the stromal cells may be genetically engineered to regulate one or more genes; or the regulation of gene expression may be transient or long-term; or the gene activity may be non-inducible or inducible. For example, one or more copies of a normal target gene, or a portion of the gene that directs the production of a normal target gene protein product with target gene function, may be inserted into human cells that populate the three-dimensional constructs using either non-inducible vectors including, but are not limited to, adenovirus, adeno-associated virus, and retrovirus vectors, or inducible promoters, including metallothionein, or heat shock protein, in addition to other particles that introduce DNA into cells, such as liposomes or direct DNA injection or in gold particles.

For example, the gene encoding the human complement regulatory protein, which prevents rejection of the graft by the host, may be inserted into human fibroblasts. Nature 375: 89 (May, 1995).

The three-dimensional cultures containing such genetically engineered stromal cells, e.g., either mixtures of stromal cells each expressing a different desired gene product, or a stromal cell engineered to express several specific genes are then implanted into the patient to allow for the amelioration of the symptoms of valvular disease. The gene expression may be under the control of a non-inducible (i.e., constitutive) or inducible promoter. The level of gene expression and the type of gene regulated can be controlled depending upon the treatment modality being followed for an individual patient.

The use of the three-dimensional culture in gene therapy has a number of advantages. Firstly, since the culture comprises eukaryotic cells, the gene product will be properly expressed and processed in culture to form an active product. Secondly, gene therapy techniques are useful only if the number of transfected cells can be substantially enhanced to be of clinical value, relevance, and utility; the three-dimensional cultures of the invention allow for expansion of the number of transfected cells and amplification (via cell division) of transfected cells.

A variety of methods may be used to obtain the constitutive or transient expression of gene products engineered into the stromal cells. For example, the transkaryotic implantation technique described by Seldon, R.F., et al., 1987, Science 236:714-718 can be used. "Transkaryotic", as used herein, suggests that the nuclei of the implanted cells have been altered by the addition of DNA sequences by stable or transient transfection. The cells can be engineered using any of the variety of vectors including, but not limited to, intergating viral

vectors, e.g., retrovirus vector or adeno-associated viral vectors, or non-integrating replicating vectors, e.g., papilloma virus vectors, SV40 vectors, adenoviral vectors; or replication-defective viral vectors. Where transient expression is desired, non-integrating vectors and replication defective vectors may be preferred, since either inducible or constitutive promoters can be used in these systems to control expression of the gene of interest. Alternatively, integrating vectors can be used to obtain transient expression, provided the gene of interest is controlled by an inducible promoter.

Preferably, the expression control elements used should allow for the regulated expression of the gene so that the product is synthesized only when needed in vivo. The promoter chosen would depend, in part upon the type of tissue and cells cultured. Cells and tissues which are capable of secreting proteins (e.g., those characterized by abundant rough endoplasmic reticulum, and golgi complex) are preferable. Hosts cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.) and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which, in turn, can be cloned and expanded into cell lines. This method can advantageously be used to engineer cell lines which express the gene protein product.

Any promoter may be used to drive the expression of the inserted gene. For example, viral promoters include but are not limited to the CMV promoter/enhancer, SV 40, papillomavirus, Epstein-Barr virus, elastin gene promoter and β-globin. If transient expression is desired, such

constitutive promoters are preferably used in a non-integrating and/or replication-defective vector. Alternatively, inducible promoters could be used to drive the expression of the inserted gene when necessary. For example, inducible promoters include, but are not limited to, metallothionein and heat shock protein.

Examples of transcriptional control regions that exhibit tissue specificity for connective tissues which have been described and could be used, include but are not limited to: elastin or elastase I gene control region which is active in pancreatic acinar cells (Swit et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515). The deposition of elastin is correlated with specific physiological and developmental events in different tissues, including the heart valves. For example, atrioventricular valve cusps are initially thick and fleshy in an embryo, and later in the development are transformed into thin and fibrous cusps. In developing arteries, elastin deposition appears to be coordinated with changes in arterial pressure and mechanical activity. Animals that contain valves and ligamental structures that are elastic contain elastin. The transduction mechanisms that link mechanical activity to elastin expression involve cell-surface receptors. Once elastin-synthesizing cells are attached to elastin through cell-surface receptors, the synthesis of additional elastin and other matrix proteins may be influenced by exposure to stress or mechanical forces in the tissue (for example, the constant movement of the construct in the bioreactor) or other factors that influence cellular shape.

Once genetically engineered cells are implanted into an individual, the presence of TPA, streptokinase or urokinase activity can bring about amelioration of platelet aggregation, blood coagulation or thromboembolism. This activity is maintained for a

limited time only, for example, to prevent potential complications that generally develop during the early phase after valve implantation, such as, platelet aggregation, blood clotting, coagulation or thromboembolism. Alternatively, once genetically engineered cells are implanted into an individual, the presence of the anti-inflammatory gene products, for example, peptides or polypeptides corresponding to the idiotype of neutralizing antibodies for TNF, IL-2, or other inflammatory cytokines, can bring about amelioration of the inflammatory reactions associated with valvular disease.

The stromal cells used in the three-dimensional culture system of the invention may be genetically engineered to "knock out" expression of factors or surface antigens that promote clotting or rejection at the implant site. Negative modulatory techniques for the reduction of target gene expression levels or target gene product activity levels are discussed below. "Negative modulation", as used herein, refers to a reduction in the level and/or activity of target gene product relative to the level and/or activity of the target gene product in the absence of the modulatory treatment. The expression of a gene native to stromal cell can be reduced or knocked out using a number of techniques, for example, expression may be inhibited by inactivating the gene completely (commonly termed "knockout") using the homologous recombination technique. Usually, an exon encoding an important region of the protein (or an exon 5' to that region) is interrupted by a positive selectable marker (for example *neo*), preventing the production of normal mRNA from the target gene and resulting in inactivation of the gene. A gene may also be inactivated by creating a deletion in part of a gene, or by deleting the entire gene. By using a construct with two regions of homology to the target gene that are far apart in the genome, the sequences intervening the

two regions can be deleted. Mombaerts, P., et al., 1991, Proc. Nat. Acad. Sci. U.S.A. 88:3084-3087.

Antisense and ribozyme molecules which inhibit expression of the target gene can also be used in accordance with the invention to reduce the level of target gene activity. For example, antisense RNA molecules which inhibit the expression of major histocompatibility gene complexes (HLA) shown to be most versatile with respect to immune responses. Still further, triple helix molecules can be utilized in reducing the level of target gene activity. These techniques are described in detail by L.G. Davis, et al., eds, Basic Methods in Molecular Biology, 2nd ed., Appleton & Lange, Norwalk, Conn. 1994.

Using any of the foregoing techniques, the expression of fibrinogen, von Willebrands factor, factor V or any cell surface molecule that binds to the platelet $\alpha 2B\beta$-3 receptor can be knocked out in the stromal cells to reduce the risk of clot formation at the valve. Likewise, the expression of MHC class II molecules can be knocked out in order to reduce the risk of rejection of the graft.

In yet another embodiment of the invention, the three-dimensional culture system could be used in vitro to produce biological products in high yield. For example, a cell which naturally produces large quantities of a particular biological product (e.g., a growth factor, regulatory factor, peptide hormone, antibody, etc.), or a host cell genetically engineered to produce a foreign gene product, could be clonally expanded using the three-dimensional culture system in vitro. If the transformed cell excretes the gene product into the nutrient medium, the product may be readily isolated from the spent or conditioned medium using standard separation techniques (e.g., HPLC, column chromatography, electrophoretic techniques, to name but a few). A "bioreactor" has been devised which takes advantage of

the flow method for feeding the three-dimensional cultures in vitro. Essentially, as fresh media is passed through the three-dimensional culture, the gene product is washed out of the culture along with the cells released from the culture. The gene product is isolated (e.g., by HPLC column chromatography, electrophoresis, etc.) from the outflow of spent or conditioned media.

6. EXAMPLE: THREE-DIMENSIONAL HEART VALVE CULTURE SYSTEM

The three-dimensional culture of the present invention provides for the growth of stromal cells such as fibroblasts upon decellularized heart valves in vitro, in a system designed to mimic physiologic conditions in vivo. Importantly, the cells replicated in this system synthesize proteins similar to those produced by the normal aortic wall and leaflet cells.

Heart valve extracellular matrix is composed mainly of elastin and collagen types I and III. The following example describes a method for growing transplantable or bioprosthetic heart valve tissue in culture by inoculating stromal cells from an exogenous source on aortic walls and leaflets, and obtaining morphologically and functionally normal human cells proliferating on the three-dimensional framework.

6.1. MATERIAL AND METHODS

6.1.1. CELLS AND PORCINE TISSUE

Porcine aortic walls and leaflets were washed with phosphate buffered saline and used fresh or after being frozen at -20°C to -70°C in sterile water or after detergent and/or enzyme extraction or any aforementioned tissue in combination with sterilization techniques as described in U.S. Patent No. 4,776,85. See Section 5.1.

Dermal foreskin fibroblasts were cultured in vitro by routine procedures. Fibroblasts used in the studies

were in their eighth passage at the time of seeding to the porcine tissues.

6.1.2. FOUR-WEEK IN VITRO CULTURES OF PORCINE AORTIC LEAFLETS AND WALLS

Porcine aortic leaflets and walls were seeded in eight well dishes with $1x10^5$ human dermal fibroblasts and cultured for one day. The aortic walls and leaflets were transferred into new well dishes and grown for an additional four weeks. The eight cultures were made up of: (1) previously frozen leaflet seeded with human fibroblasts; (2) previously frozen wall seeded with human fibroblasts; (3) previously frozen leaflet without seeding; (4) previously frozen wall without seeding; (5) fresh leaflet seeded with human fibroblasts; (6) fresh wall seeded with human fibroblasts; (7) fresh leaflet without seeding; and (8) fresh wall without seeding. The cultures were labeled with [^{35}S]-methionine and [^{35}S]-cysteine (Tran ^{35}S-Label, ICN) for four hours. The samples were boiled in Laemmli sample buffer containing β-mercaptoethanol, fractionated by SDS polyacrylamide gel electrophoresis (SDS•PAGE), and analyzed by autoradiography.

6.1.3. LESS THAN 1 WEEK TO 18-WEEK IN VITRO CULTURES OF PORCINE AORTIC LEAFLETS AND WALLS

Decellularized porcine leaflet or wall tissues were housed in a multi-well dish (one piece of tissue/well) as described above in Section 6.1.2. The human dermal fibroblasts were suspended in a nutrient-rich growth medium and were seeded onto the specific types of porcine leaflet or wall tissues such as: 1) frozen leaflets and walls; 2) electron beamed leaflets; 3) detergent and/or enzyme extracted leaflets and walls; and 4) detergent and/or enzyme extracted + electron beamed leaflets.

Each culture dish was maintained at 37°C in a sterile, static culture (no media flow) environment.

These human fibroblast-porcine tissue composites which grow in the tissue culture dish are referred to as heart valve constructs. The constructs were analyzed for:

a) **Cell Distribution**

In order to track the distribution and migration of the human dermal fibroblasts into the porcine tissue matrix, an antibody (human anti-prolyl-4-hydroxylase) (DAKO-O-Fibroblast, 5B5, Code No. M 877) was used to identify the cells post-colonization. The antibody does not cross-react with porcine tissue. See Figure 5.

b) **Cell Viability**

i) **MTT Assay:** This assay is used to assess the viability of cells after growing on the porcine matrix. Metabolically active (living) fibroblasts convert the MTT substrate (0.5mg/ml) into an insoluble purple precipitant within the cells. The purple precipitant can be visualized by the naked eye and this reflects the pattern of the viable fibroblast distribution on the porcine matrix. The MTT reaction can be quantified by measuring the optical density with a spectrophotometer (540nm) after extraction in isopropanol as described in Triglia, D., et al., 1991, Toxic. in Vitro 5:573-578.

ii) **Glucose consumption:** As an indicator of fibroblast viability, nutrient consumption (glucose) and metabolic waste products (lactate) contained in the tissue construct are measured as described in Halberstadt, C.R., et al., 1994, Biotechnology and Bioengineering 43:740-746. Viable fibroblasts decrease the concentration of glucose over time and increase the concentration of lactate.

c) **Cell Proliferation**

i) **Thymidine [^{3}H-thy] Incorporation:** Radioactive thymidine (10 μCi) is added to the nutrient-rich media during the 24-72 hr culture of tissue constructs. When

fibroblasts in the constructs divide to produce additional cells, some of the ^{3}H-thy becomes incorporated in the DNA of the cells. Excess, non-incorporated ^{3}H-thy is removed after washing the labelled constructs in 1% triton-X-100 for 2 hr and rinsing in PBS. The incorporated ^{3}H-thy can be measured using a scintillation counter.

ii) <u>BrdU Incorporation</u>: An alternative method for measuring fibroblast proliferation in tissue constructs is to add 5-bromodeoxyuridine (BrdU) to the culture media. BrdU is a non-radioactive, thymidine analog which incorporates into newly synthesized DNA of dividing fibroblasts. Tissues are incubated in BrdU-containing media for 24-48 hr. The fibroblasts containing BrdU can be visualized in the histology sections of the tissue constructs using a monoclonal antibody to the BrdU, followed by an enzyme-chromogen detection system using the Zymed Kit. (ZYMED Laboratories, Inc. San Francisco, Ca).

d) <u>Protein Assays</u>

These methods utilize radiolabelled amino acids which are added to the nutrient-rich media during the tissue culture process. The radiolabelled amino acids are incorporated into newly synthesized proteins in the tissue constructs and can be measured using a scintillation counter and/or extracted and separated on a polyacrylamide (10%) gel by their molecular weights. The gel is washed in salicylic acid (1M), then exposed to an X-ray film (4-16 hr at 4 -25°C) which, upon developing, detects the images of radiolabelled proteins.

i) <u>^{3}H-Proline Labeling</u>: Proline is a major amino acid constituent of the collagen proteins in the tissue constructs. The amount of radioactive proline incorporated (incubation in 10μCi/ml for 24-72 hr) is quantified by scintillation counting to reflect the amount of collagen being newly synthesized. Excess, non-

incorporated label is removed after washing the labeled constructs in 1% triton X-100. Ascorbic acid (25-50μg/ml) can be used as a positive inducer of collagen synthesis through activation of the propyl4-hydroxylase enzyme.

ii) ^{35}S-Cysteine/Methionine Labeling: Constructs are incubated for 0.5 hr in medium free of cysteine and methionine, then in medium containing labeled cysteine and methionine (0.2mCi/ml) for 4 hr. The radioactive isotopes are incorporated into newly synthesized proteins. The labelled tissues can then be digested in laemeli sample buffer under reducing (β-mercaptoethanol) conditions and separated by SDS-PAGE. Specific proteins can be quantified by western blotting.

e) Protein Immunohistochemistry

This method detects specific proteins in a histological section of tissue using monoclonal or polyclonal antibodies. The antibodies used specifically detect human proteins and react with: 1) human fibroblasts (prolyl-4-hydroxylase); 2) a small component of human elastin fibers (in valve wall and leaflet tissue); and 3) human tenascin (matrix glycoprotein). The antibody to the target protein is added to deparaffined or frozen sections. A second antibody which recognizes the primary antibody, is conjugated to an enzyme-chromogen visualization system.

6.1.4. IN VITRO COLONIZATION OF AORTIC WALLS AND LEAFLETS UNDER DYNAMIC CONDITIONS

Porcine leaflets were glued (medical grade cyanoacrylate) either along one surface (immobilizing the tissue) or on one edge (allowing some movement) to a polycarbonate cassette sterilized by electron beam radiation (E-beam). Human fibroblasts were seeded dynamically (5 ml/min flow rate) on these tissues and cultured for three days. The tissues were excised from

the cassette and labeled with [^{35}S]-methionine and [^{35}S]-cysteine for four hours. Tissues were boiled in Laemmli sample buffer, insoluble material was pelleted, and supernatants were fractionated by SDS•PAGE and visualized by autoradiography.

6.1.5. IN VITRO COLONIZATION OF WHOLE AORTIC VALVES UNDER DYNAMIC CONDITIONS

Whole porcine valves were either sterilized by E-beam radiation or disinfected by an antibiotic/antimycotic solution. Valves were placed in E-beam sterilized "bioreactor" and seeded with 40-50 x10^6 cells at a flow rate of 15-50 ml/min in recirculating nutrient-rich medium. After culture for up to 4 (or 8 weeks)with nutrient exchanges every week, the constructs were evaluated by MTT assay, histological staining and immunohistochemistry.

6.2. RESULTS

6.2.1. PORCINE AORTIC LEAFLETS AND WALLS

While the frozen, thawed, unseeded aortic leaflets and walls did not incorporate appreciable amounts of label (Fig. 1, lanes 3 and 4, respectively), the leaflets and walls which were seeded with human fibroblasts incorporated the radioactive amino acid precursors (^{35}S-cys/met) and synthesized proteins ranging in molecular weight from approximately 29,000 to 200,000 daltons (Fig. 1, lanes 1 and 2, respectively).

Lanes 5 through 8 describe corresponding results obtained with fresh, unfrozen leaflets and walls. Seeding fresh, unfrozen aortic leaflets and walls with human fibroblasts, resulted in an increase in the incorporation of the amount of radioactivity, in lanes 5 and 6. This increase is similar to that observed in lanes 1 and 2, respectively. Lanes 7 and 8, containing unseeded fresh, unfrozen aortic leaflets and walls,

respectively, demonstrated protein synthesis by endogenous viable cells. The aortic walls were less metabolically active than the leaflets. Of particular interest is the fact that the protein profile shown in lane 7 is similar to the protein profile in lane 1, indicating that proteins synthesized by fibroblasts seeded onto frozen porcine leaflets are similar to proteins that are synthesized by the endogenous cells of normal, fresh porcine leaflets.

6.2.2. COLONIZATION OF AORTIC WALLS AND LEAFLETS AT 8-18 WEEKS

Human dermal fibroblasts were able to colonize every tissue type from biopsies of aortic leaflets and walls over all time intervals described in Table II. The greatest fibroblast penetration of the porcine matrix occurred in leaflets, specifically in detergent and/or enzyme extracted leaflets (Figure 2). Overall, the cell distribution in the detergent and/or enzyme extracted leaflets cultured 8 to 18 weeks appeared to approach cell densities typical of a fresh porcine leaflet. Figure 3 represents cell distribution in detergent and/or enzyme extracted leaflets cultured for 4 weeks.

Cell viability assessments (MTT assay) demonstrated that the human fibroblasts remained metabolically alive even after 18 weeks. The fibroblasts were also shown to be proliferating (^{3}H-thy incorporation assay) throughout the culture process (Figure 4).

Protein production, measured as collagen synthesis (^{3}H-proline labeling) indicated that the human dermal fibroblasts were producing collagen and some proteins that are present in porcine leaflets (^{35}S-cysteine/methionine labeling) (Figure 5).

Proteins typical of heart valve tissue were identified by immunohistochemistry using specific antibodies. Fibroblasts produced human tenascin to supplement the existing porcine scaffolding (Figure 6).

6.2.3. COLONIZATION OF AORTIC WALLS AND LEAFLETS UNDER DYNAMIC CONDITIONS

Lanes 1 and 2 (Fig. 7) containing samples of porcine leaflets that were glued along their entire surface, and cultured under dynamic flow had no appreciable staining. Lanes 3 and 4 show porcine leaflets that were glued on one edge, with an appreciable amount of radioactivity was incorporated after growth for three days post seeding; when porcine leaflets were glued along an entire surface, minimal [^{35}S] was incorporated into protein. An unseeded, E-beam sterilized leaflet used as a control (lane 5) showed no incorporation of radioactivity.

Thus, porcine aortic leaflets and walls can be statically or dynamically seeded with human fibroblasts. These human fibroblasts attach and colonize the aortic leaflet and wall scaffolds, and remain metabolically active by secreting extracellular matrix molecules.

6.2.4. IN VITRO COLONIZATION OF WHOLE AORTIC VALVES UNDER DYNAMIC CONDITIONS

Human fibroblasts grew under dynamic conditions, on a porcine matrix which was previously decellularized by detergent and/or enzyme treatment. The proliferating cells were labeled with Brdu and detected using an antibody to Brdu. See Figure 8.

When the human fibroblasts were grown on a porcine matrix which was previously decellularized by detergent and/or enzyme treatment + electron beamed, the matrix seeded under dynamic, pulstile flow conditions had greater and more uniform fibroblast attachment that the matrix grown under static conditions, as shown by using the MTT assay (an indicator of cell viability as described in Section 6.1.3 (b) above). See Figure 9.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and

components are within the scope of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A living stromal cell-colonized heart valve prepared *in vitro*, comprising stromal cells and connective tissue proteins naturally secreted by the stromal cells which are inoculated upon a heart valve such that the heart valve becomes repopulated with viable cells to form a three-dimensional structure having interstitial spaces bridged by the stromal cells.

2. The living stromal cell-colonized heart valve of Claim 1 in which the stromal cells are fibroblasts.

3. The living stromal cell-colonized heart valve of Claim 1 in which the stromal cells are human dermal fibroblasts.

4. The living stromal cell-colonized heart valve of Claim 1 in which the stromal cells are human cardiac fibroblasts.

5. The living stromal cell-colonized heart valve of Claim 1 in which the heart valve is of porcine origin.

6. The living stromal cell-colonized heart valve of Claim 5 in which the heart valve is decellularized prior to stromal cell inoculation.

7. The living stromal cell-colonized heart valve of Claim 6 in which the heart valve is decellularized by enzymatic and detergent treatment or made non-viable by freezing/thawing.

8. The living stromal cell-colonized heart valve of Claim 1 in which the heart valve is composed of biodegradable material.

9. The living stromal cell-colonized heart valve of Claim 8 in which the biodegradable material comprises polyglycolic acid, catgut sutures, collagen, cellulose, gelatin, hyaluronic acid or polyhydroxyalkanoates.

10. A method for preparing a living stromal cell-coated heart valve *in vitro*, comprising culturing stromal cells inoculated onto a heart valve in a culture medium, so that the stromal cells and connective tissue human matrix proteins naturally secreted by the stromal cells attach to the heart valve to form into a three-dimensional construct.

11. The method of Claim 10 in which the stromal cells are fibroblasts.

12. The method of Claim 10 in which the stromal cells are human dermal fibroblasts.

13. The method of Claim 10 in which the stromal cells are human cardiac fibroblasts.

14. The method of Claim 10 in which the heart valve is of porcine origin.

15. The method of Claim 14 in which the heart valve is decellularized prior to stromal cell inoculation.

16. The method of Claim 15 in which the heart valve is decellularized by enzymatic and detergent treatment or made non-viable by freezing/thawing.

17. The method of Claim 10 in which the heart valve is composed of biodegradable matrices.

18. The method of Claim 17 in which the biodegradable material comprises polyglycolic acid,

catgut sutures, cellulose, collagen, gelatin, hyaluronic acid or polyhydroxyalkanoates.

19. A method of Claim 10 in which the culture medium is kept under static conditions.

20. A method of Claim 10 in which the culture medium is kept in a dynamic state by recirculating the culture medium.

21. A method of Claim 10 further comprising the use of growth factors, natural or modified blood products or bioactive molecules in the culture medium.

22. A method for transplantation or implantation of a living stromal cell-colonized heart value comprising,

(a) inoculating stromal cells on a decellularized heart valve;

(b) culturing the stromal cells so that they proliferate *in vitro* ; and

(c) implanting the stromal cell-coated humanized heart valve construct *in vivo*.

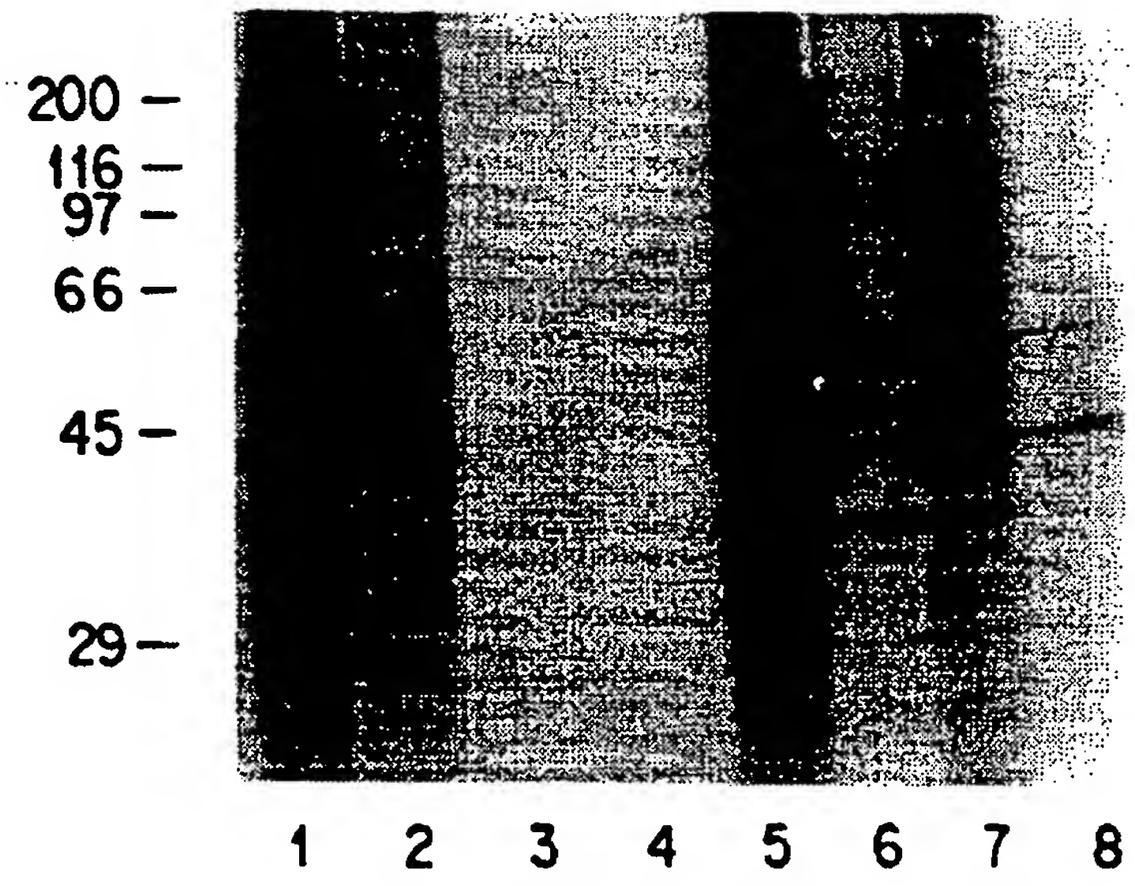

FIG. 1

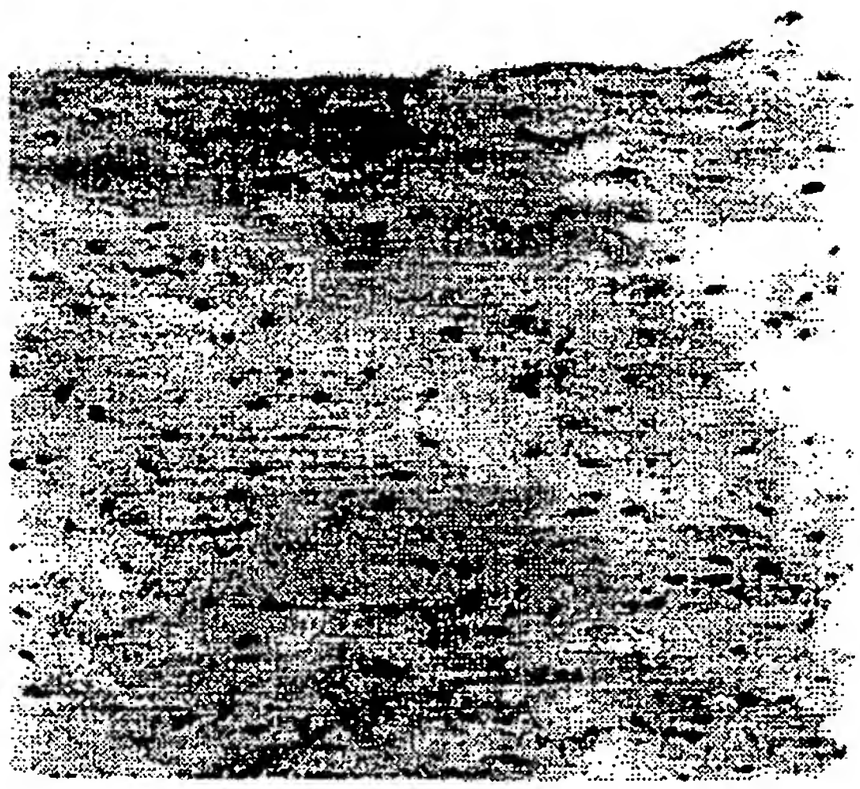

FIG.2A

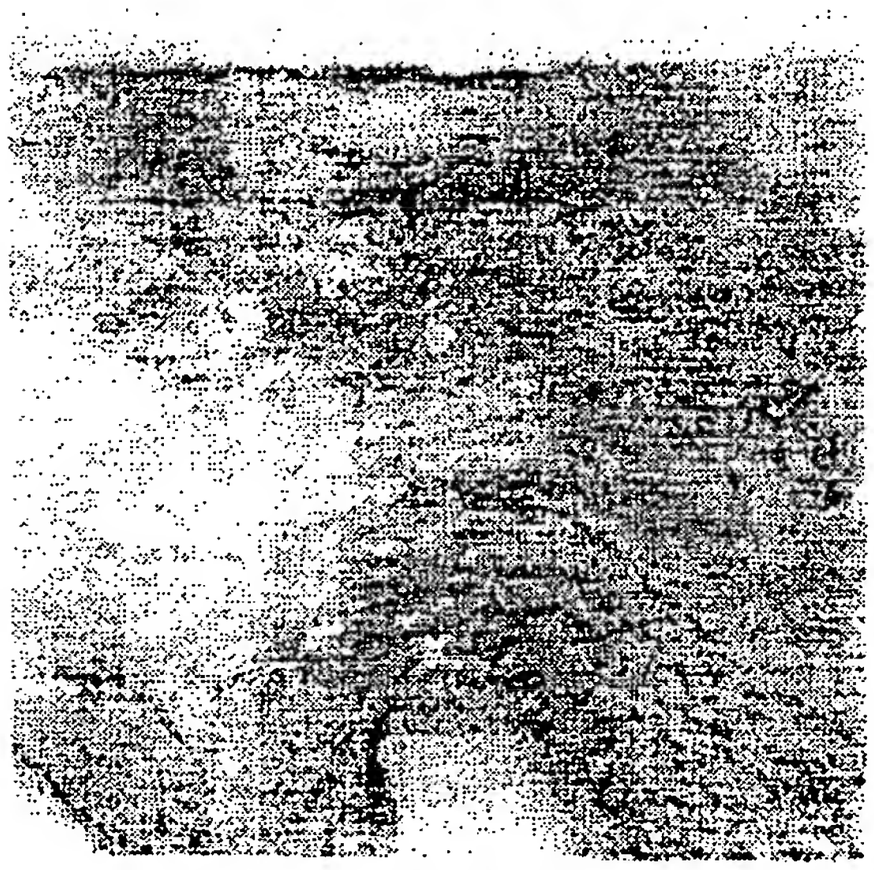

FIG.2B

FIG.2C

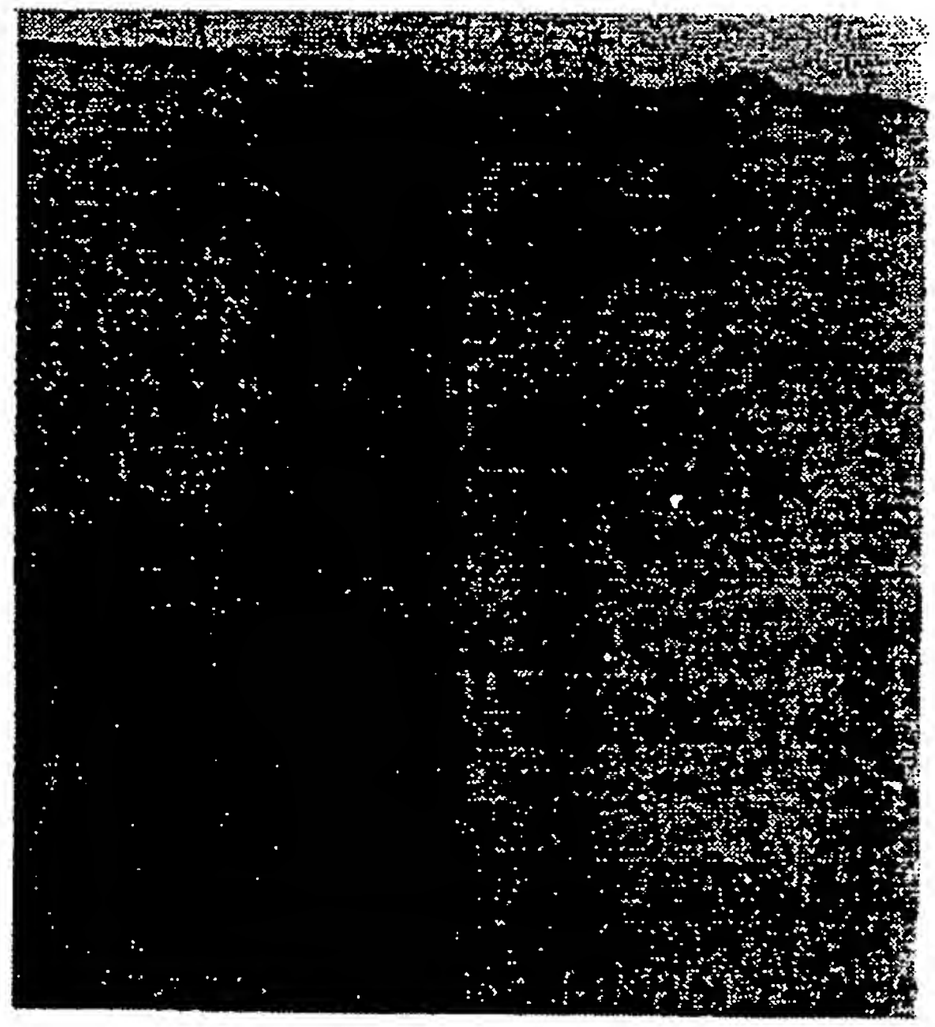

FIG.3

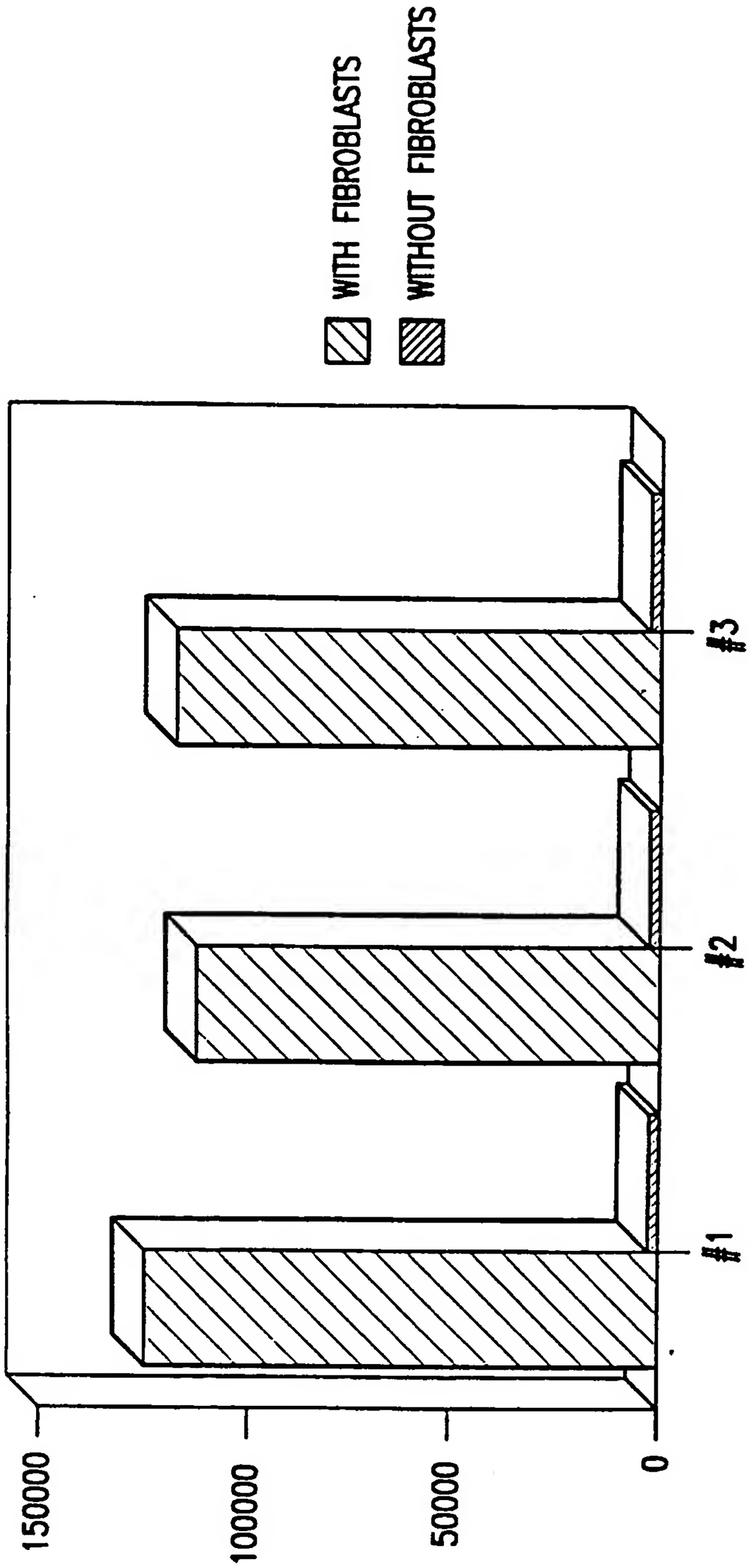
WITH FIBROBLASTS
WITHOUT FIBROBLASTS
#3
#2
#1
150000
100000
50000
0
DPM

FIG.4

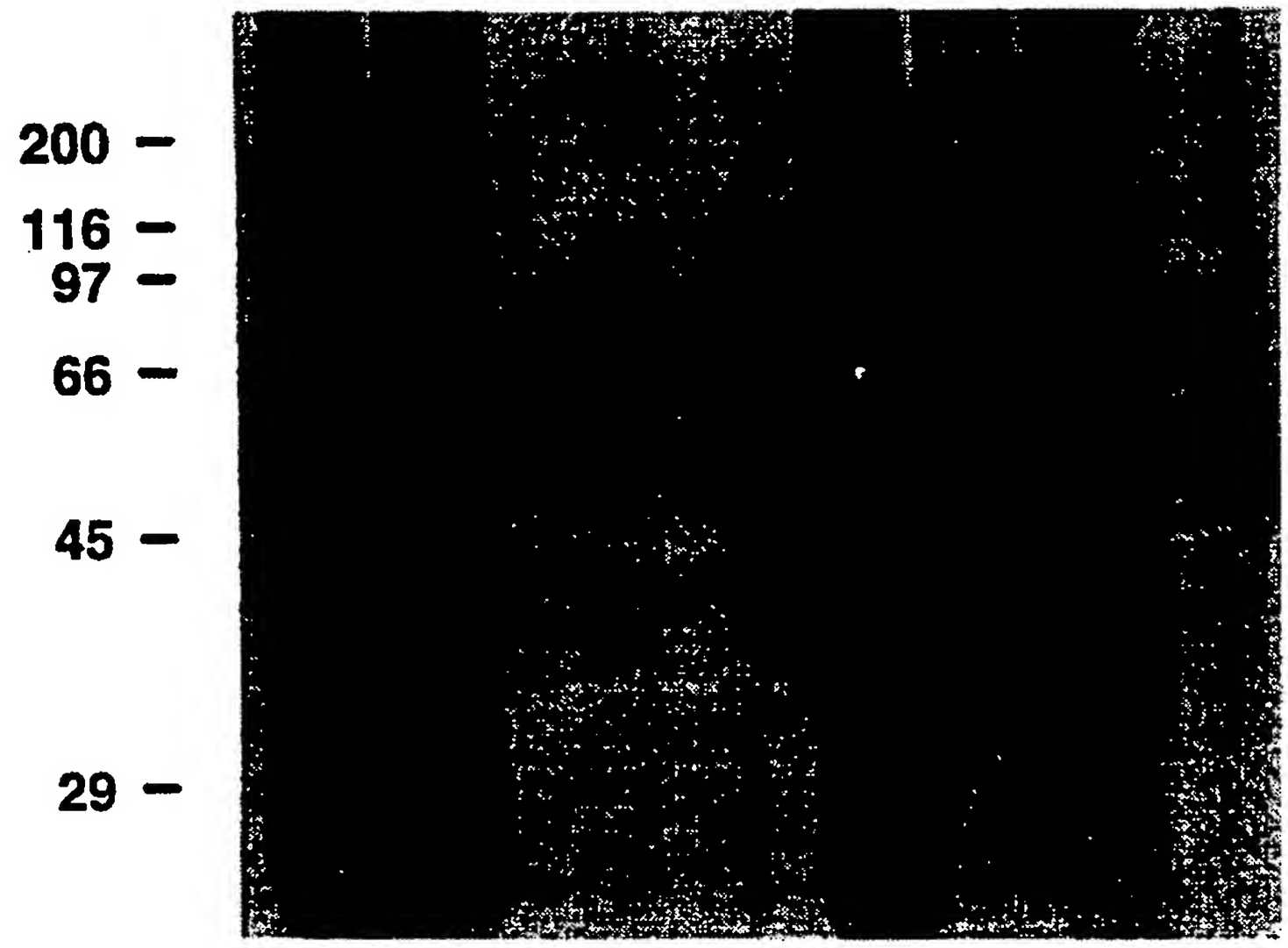

FIG.5

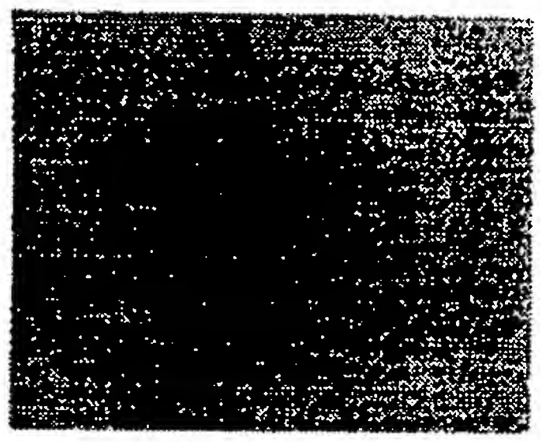

FIG.6A

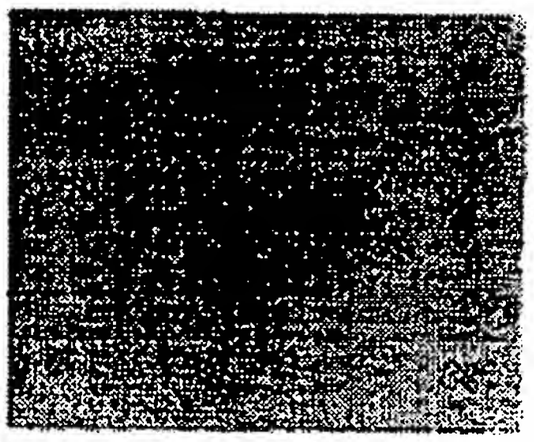

FIG.6B

FIG.6C

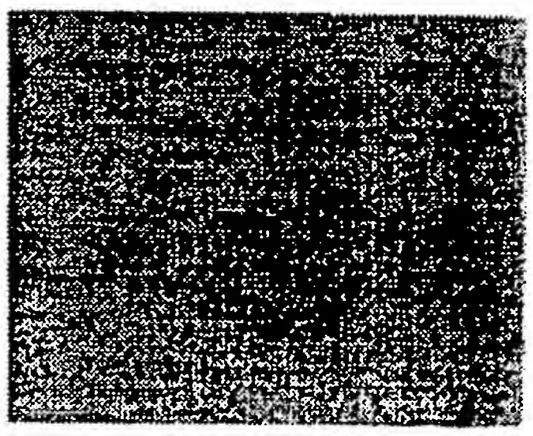

FIG.6D

FIG.6E

FIG.6F

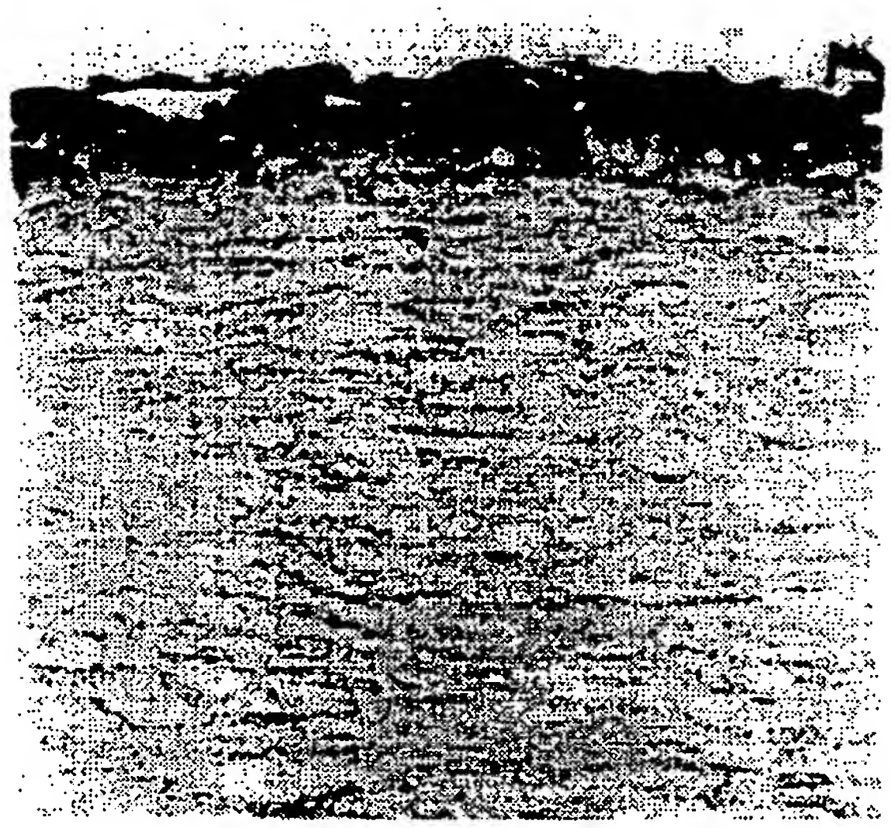

FIG.6G

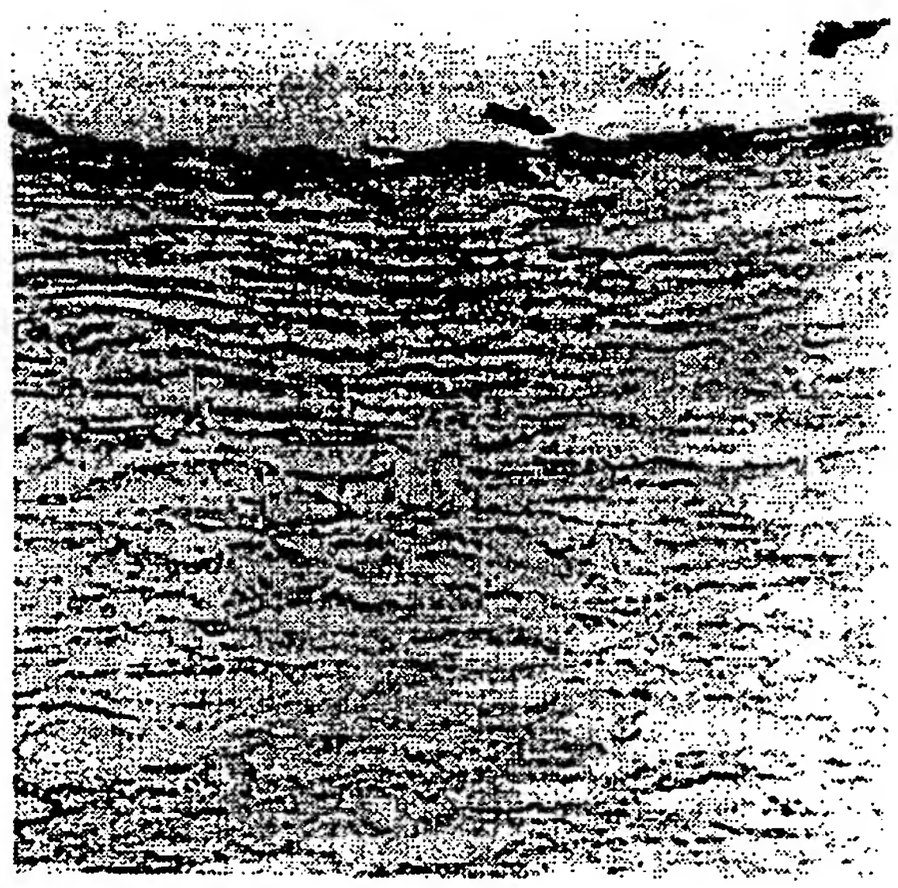

FIG.6H

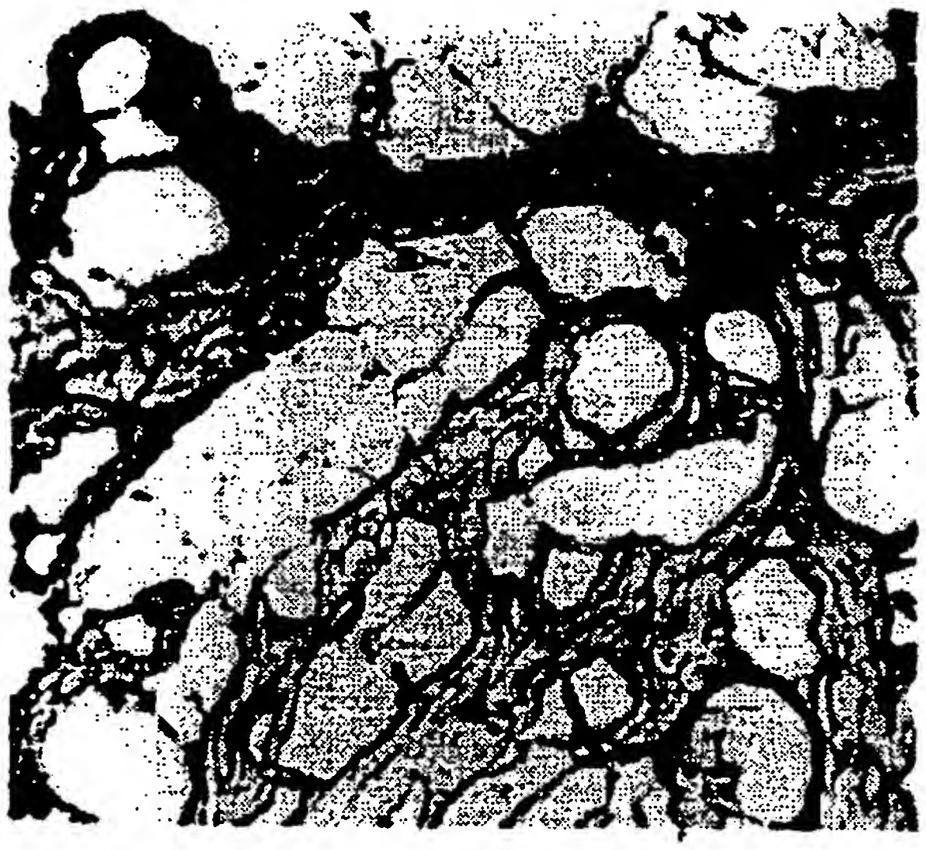

FIG.6I

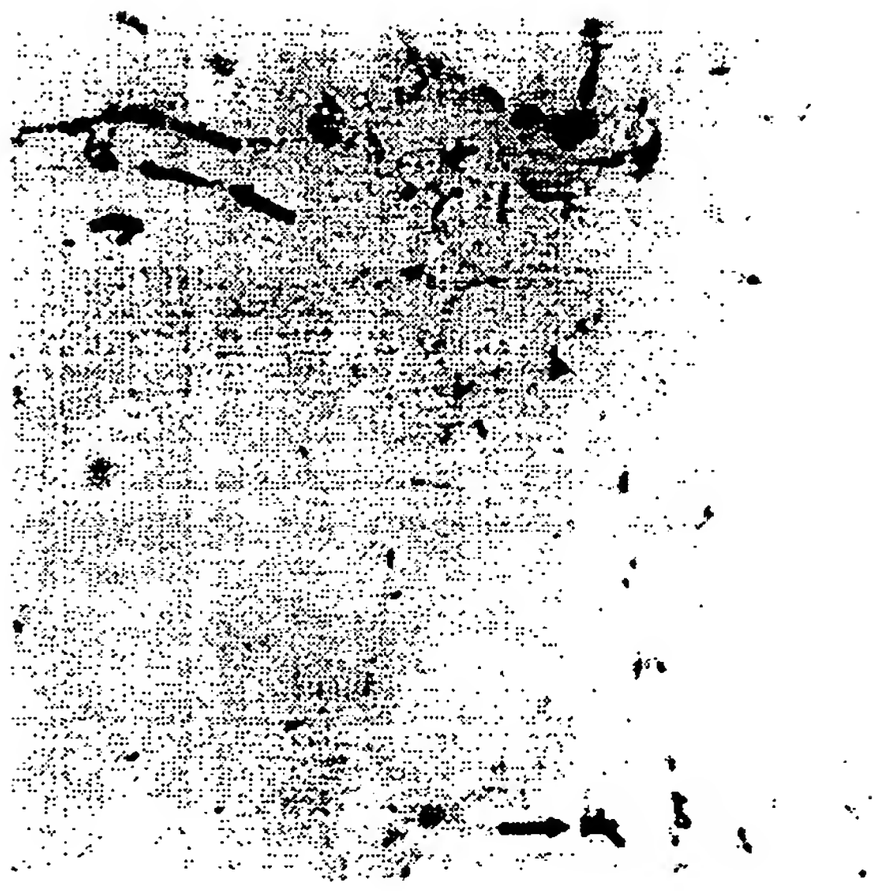

FIG.6J

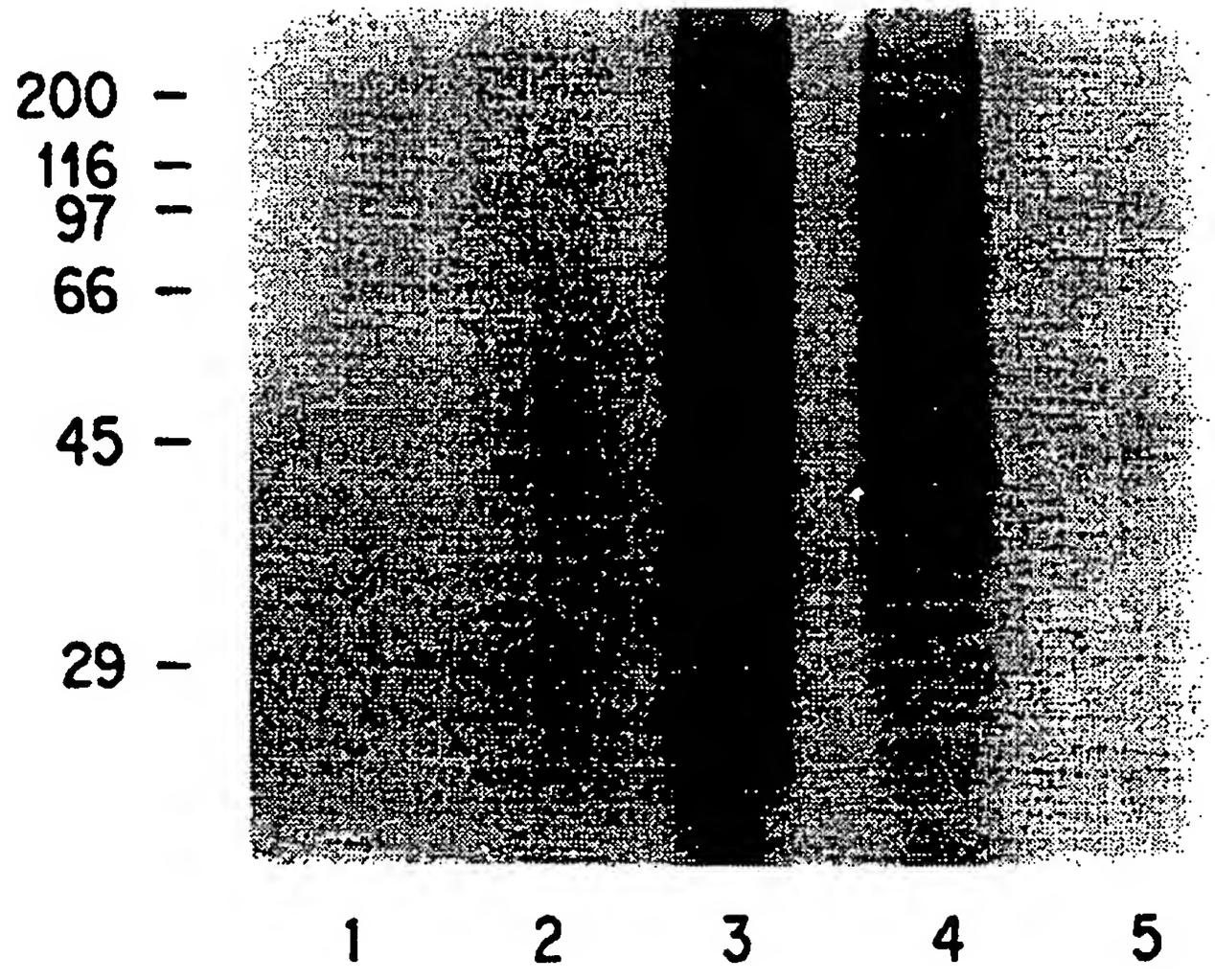

FIG. 7

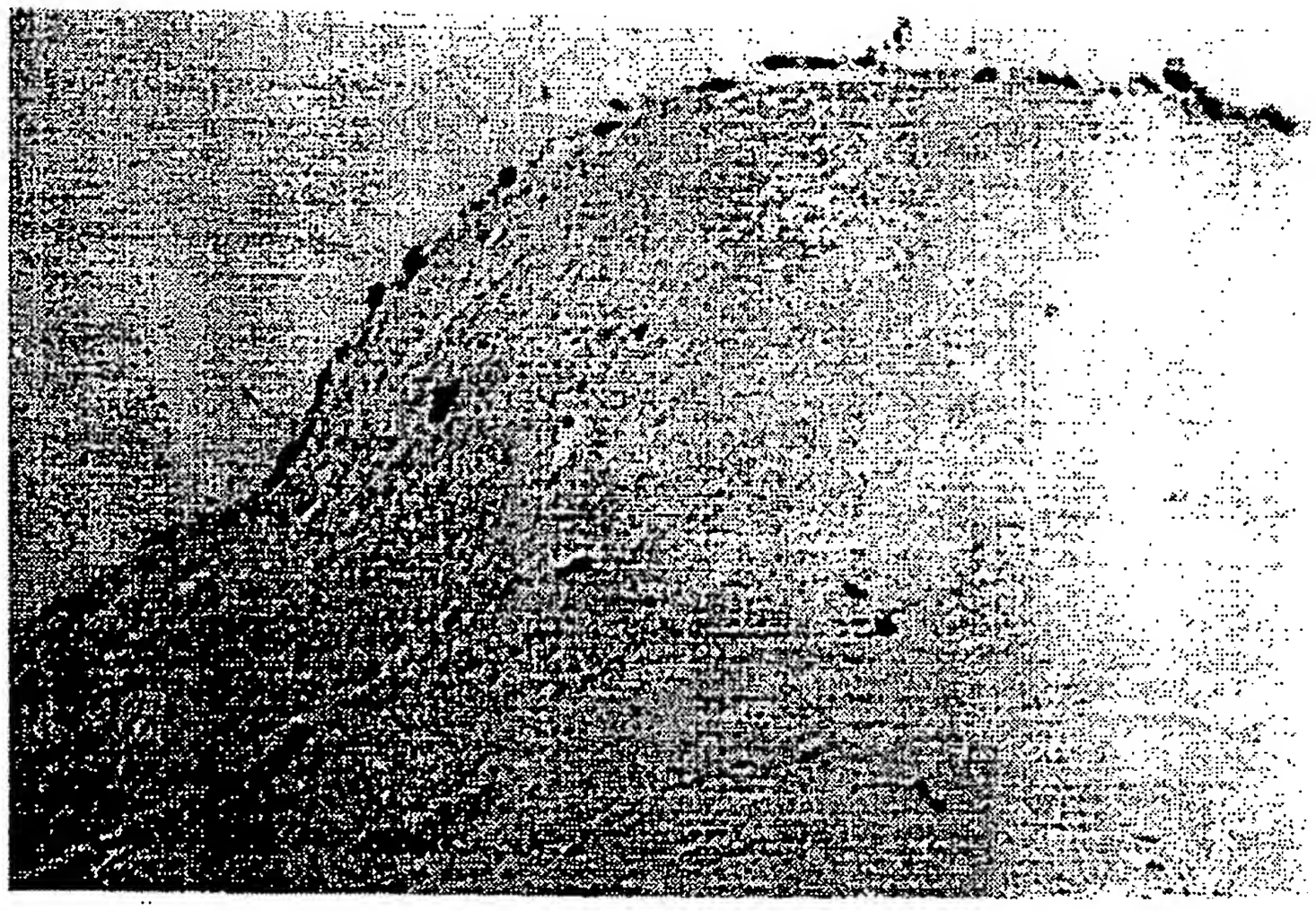

FIG.8

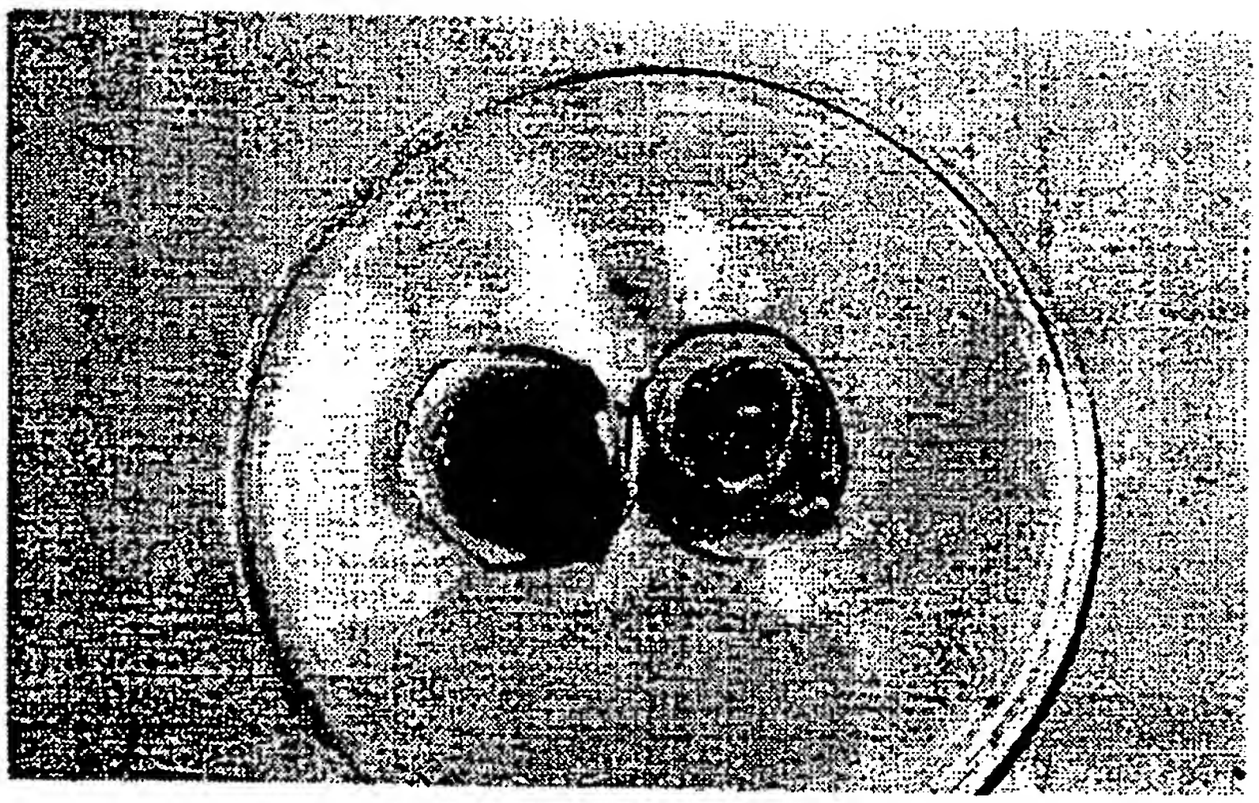

FIG.9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11395

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61F 2.24
US CL :623/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 623/2, 11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: heart valve# and stroma#(10a)cell# and fibroblast# and porcine and decelluliz?.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,192,312 (ORTON ET AL) 09 March 1993, see the entire document.	1, 2, 8-11, 17-19, and 21 --- 3-7, 12-16, 20 and 22
Y	US, A, 5,336,616 (LIVESEY ET AL) 09 August 1994, see the entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 27 OCTOBER 1995	Date of mailing of the international search report 26 DEC 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PAUL PREBILIC Telephone No. (703) 308-0858

Form PCT/ISA/210 (second sheet)(July 1992)*